Complement Autoantibodies in Atypical Haemolytic Uraemic Syndrome and IgA Nephropathy

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Abstract

Atypical haemolytic uraemic syndrome (aHUS) is renal disease associated with mutations and/or polymorphisms in genes encoding complement proteins, including complement factor H (CFH), factor I (CFI) and membrane cofactor protein (CD46). Recently, deficiency of CFH-related (CFHR) proteins 1 and 3 (*via* loss of the *CFHR3/CFHR1* gene block) was linked to the generation of autoantibodies to CFH. Around 10% of aHUS patients develop CFH autoantibodies, adding aHUS to a growing list of kidney diseases with a defined autoimmune component. IgA nephropathy (IgAN) is another such renal disease, where autoantibodies target an aberrantly glycosylated IgA1.

To investigate the role of CFH and CFHR copy number variation in the control of complement activation in aHUS and IgAN, I have first generated a full panel of recombinant CFHR proteins in mammalian cell culture. These were then used to generate unique monoclonal antibodies (mAbs) and ELISA protocols to screen for autoantibodies.

Using carefully optimised immunisation protocols, I used my recombinant CFHR proteins to produce several highly-specific CFHR mAbs. One of which targets CFHR1 (R1/1037) and three target CFHR4 (R4/244, R4/277 and R4/123). The generation of these antibodies have allowed putative ELISA screens to be developed to measure the concentration of CFHR4 in healthy individuals and aHUS patients.

My full panel of CFHR proteins also enabled screening of both aHUS and IgAN patients for the presence of autoantibodies to CFH and the CFHR proteins. Screening of aHUS plasma did not indicate the presence of any novel CFHR autoantibodies. However, IgA autoantibodies against CFHR5 (~9%) and CFH (~32%) were detected in IgAN patients. Interestingly, 64% of IgAN patients show reactivity with bovine CFH.

During this PhD, I have generated a panel of unique reagents for the study of CFHR proteins in health and disease. These have allowed me to demonstrate for the first time, the presence of CFH and CFHR5 autoantibodies in a preliminary cohort of IgAN patients.

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Dedication

To Jamie,

I would like to dedicate this thesis to you. You have been there as a special source of support throughout this whole process. Without your kindness and nurturing, this would not have been possible. For that I am eternally grateful.

Thank you for trusting me to do the best for us, and our little family…

Dino (R.I.P.), Kyle and Mooshie x

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Abbreviations

A	UV absorbance
Ab	antibody
AP	alternative pathway
APC	antigen presenting cell
aHUS	atypical haemolytic uraemic syndrome
AMD	age-related macular degeneration
amp	ampicillin
AMP	adenosine monophosphate
bCFH	bovine complement factor H
BCR	B-cell receptor
bp	base pair
BSA	bovine serum albumin
BTS	Blood Transfusion Service
C	complement component
C3NeF	C3 nephritic factor
C4BP	C4-binding protein
CCP	complement control protein
CD46	membrane cofactor protein
cDNA	complementary DNA
CHO	Chinese hamster ovary
CFB	complement factor B
CFD	complement factor D
CFH	complement factor H (human)
CFHR	complement factor H-related
CFI	complement factor I
CP	classical pathway
CR2	complement receptor 2
DC	dendritic cell
DEAP	deficiency of CFHR plasma proteins and autoantibody-positive
dH_2O	deionised water (ultra-pure)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate

DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eF1α	elongation factor 1α
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FP	forward primer
GAG	glycosaminoglycan
GalNAc	N-acetylgalactosamine
GBM	glomerular basement membrane
Gly or G	glycine
GMP	guanosine monophosphate
HAT	hypoxanthine aminopterin thymidine
HGPRT	hypoxanthine guanosine phosphribosyl transferase
His or H	histidine
HisTag	6x histidine purification tag
HRPO	horse radish peroxidase
HUS	haemolytic uraemic syndrome
Ig (M, G or A)	immunoglobulin (M, G or A)
IgAN	IgA nephropathy
IP	intraperitoneally (immunised)
kan	kanamycin
kb	kilobases
kDa	kilodalton
L5H6	5x glycine/serine linker, 6*histidine tag (GSGGGHHHHHH)
LB	Luria Bertani (media)
Leu or L	leucine
LN	lupus nephritis
LN_2	liquid nitrogen
mA	milliampere
mAb	monoclonal antibody
MAC	membrane attack complex

MBL	mannose-binding lectin
Mø	macrophage
min	minute
MLPA	multiplex ligation-dependent probe amplification
MPGN	membranoproliferative glomerulonephritis
mRNA	messenger ribonucleic acid
MW	molecular weight
neg	negative control
NFDM	non-fat dried milk
P	properdin
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	percentage hydrogen
pIgA	polymeric immunoglobulin A
PMF	peptide mass fingerprinting
PMN	polymorphonuclear (leukocytes)
pos	positive control
pro-CPB	procarboxypeptidase B
RA	rheumatoid arthritis
RCA	regulators of complement activation
rCFH	recombinant complement factor H
rCFHR	recombinant complement factor H-related
RE	restriction endonuclease
RP	reverse primer
RPMI	Roswell Park Memorial Institute (media)
RT	room temperature
SC	subcutaneously (immunised)
SCR	short consensus repeat
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second

Ser or S	serine
SLE	systemic lupus erythematosus
SN	supernatant
SNP	single nucleotide polymorphism
T4	T4 phage
TAE	tris acetate EDTA
TEMED	*N'N'N'N'*- tetramethylethylenediamine
TMA	thrombotic microangiopathy
TMBD	thrombomodulin
TMB	3,3,5,5-tetramethylbenzidine
TMP	thymidine monophospahte
Tk	thymidine kinase
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
Val or V	valine
v/v	volume per volume
w/v	weight per volume

Chapter 1

1. General Introduction

1.1 Introductory Overview

The complement system plays a diverse role in the immune response of a wide variety of different species. This highlights the ancient origins and evolution of complement[2-4]. The human complement system was initially considered as only being part of innate immunity, providing a primary line of defence against infection. However, in the last 40 years it has become increasingly evident that the complement system also plays a significant supportive role in the development of adaptive immunity[5,6].

While complement provides us with an essential first line of defence, there are occasions where problems arise and disease ensues. Disease associated with complement can be categorised into diseases associated with complement deficiency, and diseases associated with issues of complement regulation. The former constitutes a lack of complement activation, whereby the basic functions of complement are compromised leading to an increased risk from infection, while the latter results in unregulated complement activation leading to cell and tissue damage.

Complement is ubiquitous in the body and therefore protection from unwanted complement activation in all tissues and organs is required. It is not surprising that deficiencies in complement genes/proteins result in a variety of disease states. Complement has been implicated in diseases such as asthma[7-11], systemic lupus erythematosus (SLE)[12-14], glomerulonephritis[15-17], rheumatoid arthritis (RA)[18-20], multiple sclerosis[21-25], ischemia-reperfusion injury[26,27] and rejection in organ transplantation[28,29]. The complement system is also becoming increasingly implicated in diseases affecting the central nervous system, such as Alzheimer's disease and other neurodegenerative conditions[30-32].

For the purpose of this study, the emphasis will be placed upon inefficient regulation of complement activation, and this will be termed ‘dysregulation’ throughout.

Several human diseases are associated with complement dysregulation, including age-related macular degeneration (AMD), membranoproliferative glomerulonephritis type 2 (MPGN2), and atypical haemolytic uraemic syndrome (aHUS). Herein, I will focus on aHUS as a model disease to probe the complexity of complement regulation in disease. The research aims are designed specifically to bring a clearer understanding of how failure/deficiency of certain fluid-phase complement regulatory proteins may confer an increased risk of aHUS. I will also study immunoglobulin A nephropathy (IgAN), a disease where the involvement of complement is less well defined, and assess whether there is evidence that these fluid-phase complement proteins can directly influence the onset and/or progression of IgAN.

1.2 The Complement System

The bactericidal property of human serum was first described in the late 1800s[33-35]. Further studies by Ehrlich[36,37] demonstrated that this same factor was involved in the lysis of erythrocytes by immune serum. It was Ehrlich who first used the term 'complement' to indicate that this factor augmented or 'complemented' the bacterolytic and haemolytic activity of antibodies (Ab). It is now established that the complement system consists of over 40 proteins, present in fluid-phase, or in association with cell membranes. These complement proteins interact in a precise and tightly controlled manner to bring about a diverse range of effects, including the promotion of inflammation, enhancement of phagocytosis, and lysis of unprotected microbial cells.

1.2.1 The Pathways of Complement Activation

The complement system is a major component of the innate immune response, with supportive roles within the development of adaptive immunity[38,39]. There are 3 activation pathways, which are triggered *via* a range of different stimuli (**Figure 1.1**). The classical pathway (CP) is activated both by immune complexes (antigen/Ab complexes) and *via* C1q bound directly to pathogenic cell surface. The lectin (LP) pathway is activated through the binding of mannose-binding lectin (MBL) or ficolin molecules to unique saccharide residues on microbial cell surfaces, and the alternative pathway (AP) is activated in response to the unregulated cell surface of microbial cells. All complement pathways eventually converge upon the formation of C3 convertase. Subsequent downstream events lead to the production of the C5 convertase, which leads to the construction of the membrane-attack complex (MAC). The MAC is a polymeric pore which ‘punctures’ unregulated cell membranes, resulting in disruption to the osmotic balance of the cell which results in cell lysis and death.

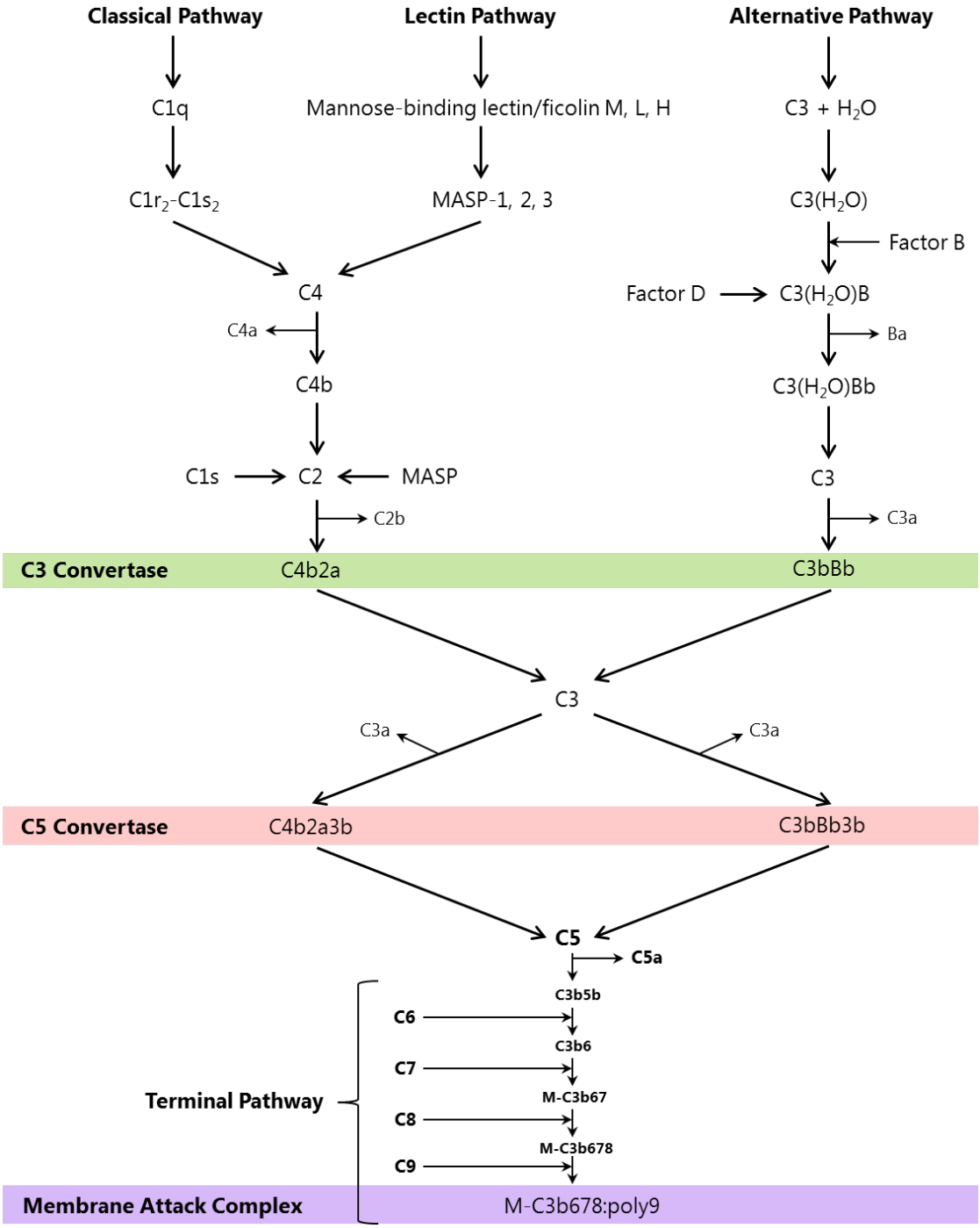

Figure 1.1: The Pathways of the Complement System

The complement system is composed of 3 pathways; the classical, the alternative, and the lectin pathway. All pathways are activated by different stimuli, but all converge to C3 convertase leading to a uniform progression to C5 convertase and the construction of the membrane attack complex (MAC).

1.2.2 The Classical and Lectin Pathways

The CP and LP pathways are activated *via* different stimuli (as previously described), but after activation the LP merges with the CP.

The CP is activated *via* the binding of the C1-complex to Ab bound antigen (i.e. immune complexes). The C1-complex comprises one copy of C1q, two copies of C1r, and 2 copies of C1s ($C1qr_2s_2$). The C1q component binds to both IgM and IgG immune complexes, causing a conformational change that leads to activation of the serine protease activity of the C1r components. C1r then cleaves C1s, activating the serine protease activity of this component. The C1s component then cleaves fluid-phase complement component 4 (C4) into C4a and C4b. The C4b fragment then associates with cell surfaces *via* covalent thioester bonding, while the C4a fragment is released with weak analphylatoxic activity[40]. C2 is recruited to the cell surface-bound C4b, and is subsequently cleaved by C1s into C2a and C2b. The C2a fragment remains in association with C4b, building the CP C3 convertase, C4b2a. The C2b fragment is released. The function of fluid-phase C2b is unclear, but it has been suggested to increase vascular permeability, thus contributing to the proinflammatory environment and infiltration of immune cells[41] The C3 convertase then recruits fluid-phase C3, cleaving it into C3a and C3b. The C3b fragment remains in association with the C3 convertase, forming the CP C5 convertase (C4b2a3b). The C3a fragment is released into fluid-phase where it serves as a potent anaphylatoxin[42]. C5 convertase then cleaves fluid-phase C5 into C5a and C5b. C5b associates with fluid-phase C6 and C7 (C5b-7) at the cell surface. C5b-7 then binds C8 to form the C5b-8 complex, which results in sequential binding of multiple copies of C9, to form the MAC[43,44]. The MAC is a membrane-spanning lytic pore which results in disruption to the osmotic balance of the targeted cell and lysis[45,46].

The LP relies upon the presence and recognition of bacterial polysaccharides by a group of collectins and ficolins with carbohydrate recognition domains. The collectin MBL, was once thought to be the only activator of the LP, but more recently a group of ficolins known as filcolin-M (or ficolin-1), ficolin-L (ficolin-2), and ficolin-H (ficolin-3) were also found to be potent activators of the pathway[47]. A recent study found another collectin with LP activatory properties, known as

CL-11[48]. Each lectin can bind to bacterial polysaccharides, and other glycoconjugates rich in D-mannose, N-acetyl-D-glucosamine and L-fucose.

Three MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3) are found associated with the lectin recognition molecules. MASP-2 is thought to be the main activator component of the LP, with MASP-1 and MASP-2 playing other up- or downregulatory functions[49-54]. Additionally, the protein products of *MASP* transcript alternative splicing (sMAP and MAP-1) are also found in association with the lectin/MASP complex, but their functional significance remains unclear.

Upon binding of the lectin component to microbial carbohydrate residues, MASP components sequentially cleaved into active serine proteases. The activated lectin/MASP complex then cleaves C4 and C2, in the same manner as the C1-complex. This leads to construction of the CP C3 convertase (C4b2a) and subsequent downstream events towards terminal MAC formation.

1.2.3 The AP of Complement Activation

The AP of complement activation is the only pathway to exist in a constitutively activated state. Approximately 1% of fluid-phase C3 exists in a hydrolysed form ($C3.H_2O$), which enables binding of complement factor B (CFB), which in-turn initiates activation of the AP. This state of immunological 'readiness' is referred to as the 'tick-over' effect. However, in steady state conditions (i.e. no microbial infection), complement activation is closely monitored by a series of regulatory proteins which act to decay $C3.H_2O$/CFB interactions. This maintains a balance between protective and destructive activation of the AP.

In the presence of an activator surface (unregulated microbial cell membranes) there is enhancement of AP complement activation. However, host tissues are protected from complement attack by a tightly controlled mechanism of complement regulation. Binding of $C3.H_2O$ to CFB is not regulated at the microbial cell surface, resulting in the proteolytic cleavage of bound CFB by fluid-phase protease complement factor D (CFD), into C3-associated Bb, and free Ba. The $C3.H_2O$/Bb complex is the first active C3 convertase in AP activation, and functions to cleave inactive C3 into C3a and C3b. C3b interacts with unregulated cell surfaces *via* thioester covalent bonding (as C4b does). As

with $C3.H_2O$, C3b complexes with CFB and is cleaved in the same manner to form an alternate version of C3 convertase, C3b/Bb. This process continues, rapidly creating a positive amplification-loop of complement activation *via* C3 convertase formation and C3 cleavage (**Figure 1.2**).

C3b/Bb also recruits an additional C3b molecule at the cell surface resulting in a $C3b_2$/Bb complex. $C3b_2$/Bb is the C5 convertase of the AP, and functions to cleave inactive C5, into the active C5b and C5a components. C5b associates with the unprotected microbial cell surface, and triggers formation of the MAC (as previously described). C3a and C5a are potent anaphylatoxins with roles in promoting inflammation, recruitment of polymorphonuclear (PMN) cells and activation/modulation of dendritic cells (DC), macrophages (Mø) and T-cells[55].

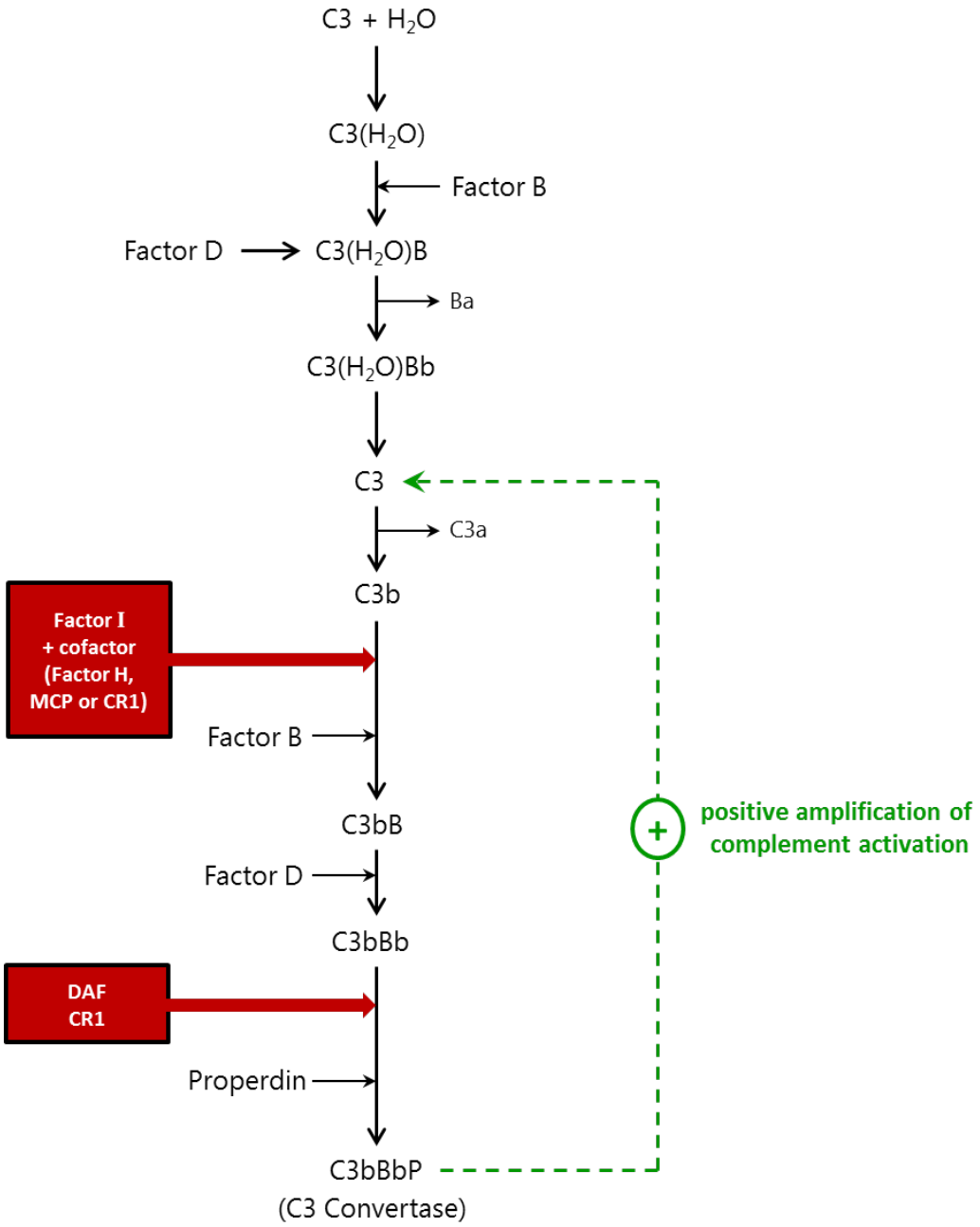

Figure 1.2: The AP of complement activation

The AP is constitutively activated due to hydrolysed C3 'tick-over'. Therefore, pathway activation is closely regulated by complement regulators (see red boxes) at the host cell surfaces. On unregulated surfaces (e.g. a bacterial cell surface), AP activation progresses, resulting in a positive amplification of self-perpetuating complement activation.

1.2.4 Regulation of AP Activation

Binding of C3b at the cell surface is indiscriminate between host tissue and pathogenic cells, so there is clearly a requirement for regulatory proteins at the host cell surface. The genes coding the complement regulatory proteins are grouped together in the 'Regulators of Complement Activation' (RCA) gene cluster, positioned on chromosome 1 (1q32), along with a selection of other immunologically active genes (**Figure 1.3**). One of the key features of the regulatory proteins encoded by genes within the RCA cluster is their unique structural presentation. They are composed of a series of short consensus repeats (SCR), also referred to as complement control proteins (CCP), or sushi domains. For simplicity, they will be referred to as SCR domains throughout.

Many of the regulatory proteins are membrane bound, and function to specifically prevent activation at the cell surface. For example, membrane cofactor protein (CD46) and complement receptor 1 (CR1) bind active C3b, allowing for fluid-phase regulator complement factor I (CFI) to cleave the complement proteins into an inactive state. Another example of a membrane-associated regulator is decay-accelerating factor (DAF). The regulatory role of DAF is to decay the AP C3 convertase (C3bBb), via specific binding to the Bb component[56]. The decay of the C3 convertase results in blocking of the AP positive amplification loop.

There are also fluid-phase regulators that circulate to prevent fluid phase activation of complement. Some fluid-phase complement regulators are thought to also regulate cell surface complement activation. Complement factor H (CFH) is a fluid-phase complement regulator that is recruited to the cell surface upon deposition of C3b (a marker of complement activation). CFI is another fluid-phase regulator, which works in conjunction with CFH, CR1 and CD46. In the AP, CFH and CFI are the main regulatory proteins which work in unison to prevent complement activation.

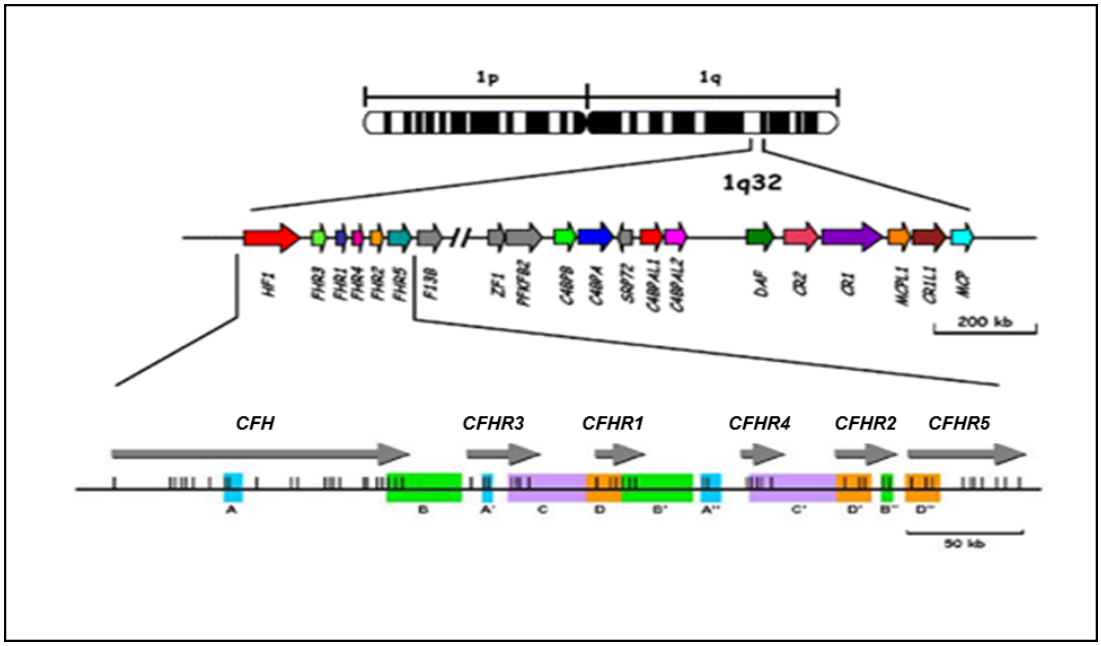

Figure 1.3: The Regulators of Complement Activation (RCA) gene cluster

The RCA gene cluster is found located on chromosome 1, at position q32. There are several complement regulatory genes found clustered here, including the genes encoding the CFH and CFHR1-5 proteins.

1.3 Complement and Adaptive Immunity

The breakdown of complement proteins during complement activation (and regulation) results in several bioactive complement protein fragments, or by-products. These include the C3a and C5a anaphylatoxins as well as the C3b/C3d opsonins. These proteins have been shown to be critical in the optimal function of the immune system as a whole. Antigen presenting cells, such as Møs and DCs, express significantly higher levels of C3a and/or C5a receptors (R) on their cell surface. Recent studies have shown that C3aR and/or C5aR are expressed on several DC subsets, in response to different stimuli (e.g. microbial antigen, inflammatory mediators, complement products)[57-61]. Therefore, C3a and C5a have the potential to alter the differentiation and/or activation profile of DCs, which in turn will selectively drive a particular T-cell mediated response. Indeed, early studies into the role of C3a and C5a in adaptive immunity have highlighted that both anaphylatoxins have immune regulatory functions[62-64]. C3a has been shown to be a potent suppressor of antigen-specific and non-specific Ab responses, while C5a was found to influence both Ab responses and antigen-induced T-cell proliferation. In recent studies, several animal models have been used to demonstrate that blocking the ability of C5a to interact with the C5aR is sufficient to impair T-cell responses (including Th1[65-67], Th2[68-70] and Th17[71-73] responses).

The immune system is highly interlinked and this is never clearer than when looking at the interaction of B and T-cells. Productive T-cell responses are necessary for full activation of B-cells, as primed B-cells (encountered antigen and presenting peptides on cell surface HLA molecules) require T-cell help to become fully activated. As C3a and C5a are involved in B and T-cell responses, problems or alterations in complement activation/regulation may result in a poor adaptive immune response, or even the onset of autoimmunity.

Systemic lupus erythematosus (SLE) is a prime example where complement activation and the presence of complement deposition products are associated with disease. Beyond the strong up-regulation of C3aR in lupus prone mice with active nephritis, the C3 fragment, C3d and the function of its receptor (complement receptor 2, or CR2; expressed on B-cells), are particularly linked with disease. C3d is the final breakdown product of C3 and is covalently linked

to antigen during complement activation. If the C3d coated antigen is recognised by a specific B-cell receptor (BCR) it can also interact with CR2, resulting in the cross-linking these B-cell surface receptors. This leads to a much stronger intracellular B-cell activation signal, compared to the relatively weak signal achieved to an antigen without C3d. Therefore, complement (C3d) and CR2 play a critical role in B-cell response[74]. A key feature in SLE is low expression levels of CR2. Subsequent studies using murine models have demonstrated that abnormalities in CR2 lead to an SLE-like disease. These studies provide a rational for C3d and complement activation being regarded as essential in helping to define immune tolerance. Immune tolerance or the ability to discriminate between self and non-self is one of the biggest challenges faced in the development of a fully functional adaptive immune system.

Like many diseases with a strongly defined complement component, SLE is characterised by the deposition of immune complexes in multiple organs of the body, including the kidney, where patients are reported to present with lupus nephritis (LN). Key features in the progression of LN, are deposits in the kidney and proliferation of the mesangium. Such characteristics are also seen in IgA nephropathy (IgAN), where IgA-based immune complexes are found deposited within the mesangium. Here, the role of complement remains much less clear but evidence is building.

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1.4 Overview of the pathogenesis of IgAN

Immunoglobulin (Ig) A nephropathy (IgAN) was first described in 1968, and is now recognised as the world's most common glomerulonephritis[75,76]. IgAN is characterised by IgA1 deposition in the glomerular mesangium, with co-localised C3 deposits, and immune-complexed IgG and IgM[75,77-81]. There is also a marked increase in circulating IgA levels in over 50% of patients[82-84]. The key features of the IgA1 found increased in circulation and in mesangial deposits are that it is polymeric (pIgA1) and contains aberrantly glycosylated side chains (galactose deficient *O*-linked glycans)[85-88] that appear to influence proliferation of the mesangial cells[85].

1.4.1 The molecular basis of aberrantly glycosylated IgA1

IgA1 contains a 17 amino acid hinge region, located centrally in the α-heavy chain. The hinge region undergoes post-translation modification whereby 6 *O*-linked glycan chains can be added (**Figure 1.4**)[89]. The primary glycan modification is N-acetylgalactosamine (GalNAc), in *O*-linkage with hinge serine or threonine residues. Under normal conditions a galactose residue is attached to the GalNAc residue by the enzyme 'C1GalT1', which requires the molecular chaperone 'Cosmc' to ensure its correct folding. Therefore, an issue with either the enzyme or chaperone system could be responsible for the galactose deficient status of aberrantly glycosylated IgA1 in IgAN. Several sets of results have suggested down regulation of 'C1GalT1' and/or 'Cosmc' as a key event in this process[90-92].

1.4.2 IgAN, autoantibodies and complement activation

IgAN is now considered an immune-complex disease. The aberrantly glycosylated IgA1 is one feature of the immune complexes, but there is also evidence of autoantibodies targeting the abnormal pattern of glycosylation at the hinge region. IgM, IgG and IgA1 autoantibodies have been identified in mesangial deposits[93,94]. Breakdown products of C3 are also found co-localised with these immune complexes, suggesting complement activation and localised inflammation as another aspect of IgAN pathogenesis. The AP and MBL pathways have both been implicated in glomerular injury in IgAN[95-99].

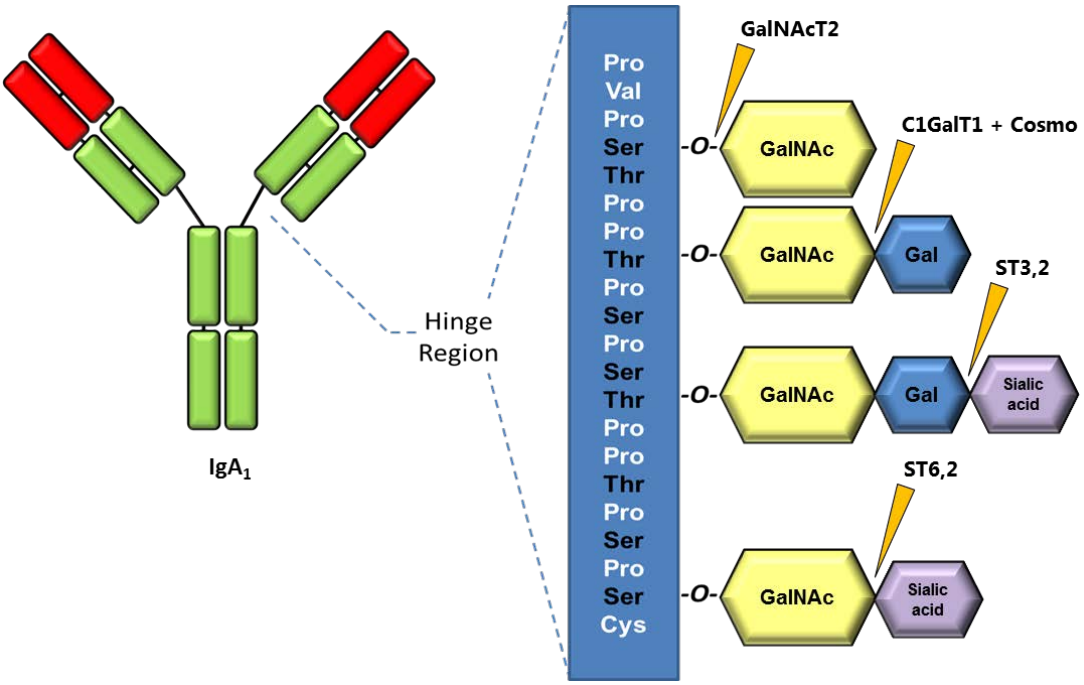


Figure 1.4: IgA1 is aberrantly glycosylated in IgAN

IgA1 undergoes *O*-linked glycosylation at serine or threonine residue at the α-heavy-chain hinge region. Glycosylation begins with the addition of *O*-linked GalNAc *via* the action of GalNAcT2. Galactose residues are then added *via* the action of C1GalT1 and its molecular chaperone Cosmc, to ensure correct folding and stability. In aberrantly glycosylated IgA1 It is unclear whether galactose is sialylated during healthy glycosylation by ST3,2. In aberrantly glycosylated IgA1, sialic acid residues may be added directly to GalNAc via the action of ST6,2, in circumstances where galactosylation is deficient. Evidence suggests sialylation may prevent the addition of galactose to this glycosylation profile. It is the absence of galactose, and presence of sialylation that is reported in IgAN.

Diseases associated with complement dysregulation are often found to result in some form of glomerular injury, as previously described with aHUS and MPGN2. This suggests a potential for a complement dysregulation in IgAN, where glomerular injury occurs and complement deposition is found. There has been some evidence to suggest a potential role of regulators of the AP in IgAN[100,101]. Additionally, certain HLA alleles have been linked with increased risk of developing IgAN[102-104]. This implicates an adaptive immune response in this disease.

Complement activation is undoubtedly involved in glomerular injury in IgAN. The localised deposition of complement proteins in the mesangium was at first poorly understood; as deposited IgA1 was considered ineffective at triggering complement *via* the CP activation. Subsequently, IgA1 has been shown to activate the AP[97]. Additionally, CP complement activation is expected with the presence of IgG or IgM autoantibodies against aberrantly glycosylated IgA1 containing immune complexes. Therefore, it may be that complement is merely being activated under normal circumstances; with detrimental secondary effect to patients with IgAN.

With the increasing evidence of complement dysregulation in association with a number of kidney diseases (including aHUS and MPGN2), IgAN is a prime candidate for screening for deficiency in complement genes/proteins. Deficiency of CR1 has been linked with IgAN, suggesting complement dysregulation could be an important factor in the pathogenesis in IgAN[105]. In order understand the complexity of complement dysregulation in disease, the pathogenesis of aHUS will now be discussed in detail.

1.5 Haemolytic Uraemic Syndrome

Haemolytic Uraemic Syndrome (HUS) is a renal disease, and one of the thrombotic microangiopathies (TMA), characterised by the presentation of haemolytic anaemia, thrombocytopenia and acute renal failure. It was first described and characterised in 1955 by Conrad Von Gasser, where he encountered five patients with renal failure, who also presented with thrombocytopenia and haemolytic anaemia[106].

HUS is often described after an enteric infection with verocytotoxin-producing strains of *Escherichia coli*, or Shiga-toxin secreting *Shigella* species[107-111]. Verocytotoxin is known as Shiga-like toxin due to the structural and functional similarities with Shiga-toxin. There are other strains of bacteria that carry the toxin, as the gene originates, and is readily transmitted by a bacteriophage[112-115]. In all infected bacteria, this toxin becomes the primary virulence factor. Verocytotoxin is a 2-part cytotoxin designed, to break through the gut epithelial barrier, inhibit protein synthesis and ultimately allow infection to enter the bloodstream. The toxin causes damage to the glomerular endothelium, resulting in platelet activation and the initiation of the coagulation cascade. This results in thromocytopenia, the formation of localised thrombi in the renal microvasculature, and damage to erythrocytes (haemolytic anaemia). Patients affected with HUS generally complain of an associated infection, generally giving rise to diarrhoea, so the disease is also referred to as diarrhoea positive HUS (d+HUS). This aids in distinguishing it from the 5-10% of HUS cases that present with symptoms of haemolytic anaemic, thrombocytopenia and renal failure, but show no evidence on enteric infection or diarrhoeal illness[116]. This group of patients are referred to as having ‘atypical’ HUS (aHUS).

1.5.1 aHUS

When aHUS was first being described, causative factors behind the disease were unknown. However, there was evidence of a hereditary component as members of the same family were being hit by the disease, often generations apart[117]. This became known as familial aHUS. It also emerged that the disease was presenting randomly within the population. This is referred to as ‘sporadic’ aHUS. The terms familial and sporadic are commonly used to categorise aHUS

patients, and are essentially terms to describe the origin of the components that contribute toward disease onset and progression.

The presentation of aHUS is strongly associated with complement dysregulation, resulting from a range of abnormalities with complement regulatory and activator proteins. In particular, problems involving the AP of complement activation. From a genetic perspective, mutations in complement genes are found in ~51% of aHUS patients[118]. In the past 20 years, several complement components have been identified as posing an increased risk of developing aHUS. The complement genes/proteins shown to predispose to aHUS will now be addressed.

1.5.1.1 The role of CFI in aHUS

CFI is fluid-phase regulator of complement activation. It functions to inactivate surface bound C3b and C4a, thus preventing progression of the C3 convertase amplification loop. CFI exists in an active state in the circulation, but requires a different compliment of cofactors to inactive each of these convertase components. AP C3b inactivation requires CFH as a cofactor, and C4b requires C4-binding protein (C4BP). CD46 serves as a cofactor for inactivation of both components. Therefore, CFI serves as complement regulator across all activation pathways, and is integral for permanent inactivation of convertase components C3b and C4b. A 2008 study was able to characterise mutations in the serine protease domain of the light chain of CFI (CFI is composed of a heavy and light chain), which resulted in a lack of C3b and C4b cofactor activity[119]. Additionally, they found a mutation leading to an issue with protein secretion. There was also a risk-associated mutation located in the heavy chain. However, the exact function of the heavy-chain domain is unclear, so how the mutation affects the function of CFI is unknown. Mutations in *CFI* are found in ~17% of aHUS patients[119]. Those with *CFI* mutations respond poorly to plasma therapy[118].

1.5.1.2 The role of CD46 in aHUS

CD46 is widely expressed on the surface of all cells, with the exception of erythrocytes. CD46 serves as a cofactor for CFI to inactivate surface bound C3b and C4b, thus regulating the AP and CP of complement activation. *CD46*

mutations are present in ~15% of aHUS patients, however despite this high frequency (relative to mutations in the other complement regulatory proteins), *CD46* mutations offers the best prognosis. In particular, after renal transplantation, those with a *CD46* mutation rarely experience a recurrence of aHUS[118].

1.5.1.3 The role of CFB in aHUS

CFB is a fluid-phase zymogen that carries the convertase protease catalytic subunit (Bb) required for activation of the AP C3 convertase. Mutations on *CFB* are present in less than 3% of aHUS patients[120]. Functional analysis of mutants suggests some *CFB* mutations confer an increased level of resistance to the decay of C3 convertase, which is because complement regulators cannot bind as efficiently to exert their decay-accelerating activity.

1.5.1.4 The role of C3 in aHUS

C3 is the central component of complement activation, enabling the construction of the C3 convertase and the pivotal downstream production of the positive amplification loop of activation[121]. Mutations in *C3* have been described in ~10% of aHUS patients[118,122]. In functional analysis of these mutations, five resulted in inefficient binding of complement regulators so were gain of function mutations. However, there were two mutations resulting in a decrease of C3 secretion, but the mechanism of which is unclear[122].

1.5.1.5 The role of thrombomodulin in aHUS

Thrombomodulin (TMBD) is a glycoprotein located on the surface of endothelial cells, is a key component of the protein C anticoagulation pathway. It functions as a cofactor in the thrombin-induced activation of protein C. Additionally, it functions to enhance thrombin-mediated activation of an inhibitor of fibrinolysis, procarboxypeptidase B (pro-CPB). Therefore, under normal conditions, TMBD functions to regulate blood coagulation *via* these two regulatory processes. When activated by TMBD, pro-CPB also acts to inactivate anaphylatoxins C3a and C5a, thus counteracting the proinflammatory actions of these complement components. TMBD has been shown to regulate the AP by binding to cell

surface bound C3b, and accelerating the cofactor activity of CFH or C4BP, in the presence of CFI.

In a recent study, the *THBD* gene was screened in 152 aHUS patients, and compared to a group of normal controls. Six different heterozygous missense mutations were found in 7 unrelated patients[123]. Functional analysis using cell coated with mutant TMBD displayed a diminished capacity to either inactivate C3b, or activate pro-CPB. The net result was less protection against complement mediated cell damage. Mutations in the gene encoding TMBD have also been shown to result in a loss of cofactor activity.

1.5.1.6 The role of CFH in aHUS

CFH is a 155 kDa single-chain serum glycoprotein, primarily secreted by the liver. CFH is the principle regulator of the AP. The circulating concentration ranges from 200-800 µg/ml[124], but how circulating levels are maintained within this homeostatic range is currently unknown. CFH is found ubiquitously throughout the body, both in circulation and in tissue interstitium to facilitate rapid regulation of the AP 'tick over'.

The *CFH* mRNA transcript is composed of 23 exons, which are translated into a chain of 20 domains, known as short consensus repeats, or SCR domains. Each SCR domain consists of ~60 amino acids, each containing two disulphide bonds between cysteine residues. Upon translation, the pro-protein is led by an 18 amino acid N-terminal signal peptide, which is cleaved prior to secretion. To-date, only the secondary structure of individual or small fragment groups of SCR domains have been investigated. However, based upon these structural and functional data, a hypothesis has been proposed as to how CFH chain may arrange at the cell surface during recruitment by surface bound C3b[125].

CFH has two main binding domains for C3b, in SCR domains 1-4, and domains 19-20[125-127]. The regulatory domain lies at the N-terminal end of the extended CFH chain, and is responsible for binding C3b to promote decay-acceleration of C3 convertase (C3b/Bb), and the cofactor activity for CFI-mediated inactivation of C3b[128-131]. The other C3b binding region lies at the opposite end of the chain (SCR domains 19-20), which binds to C3b with a high degree of affinity. It is well documented that SCR domains 19-20 also bind to polyanionic structures

such as glycosaminoglycans (GAGs) like heparin sulphate, and also to sialic acid residues in proteoglycans, glycoproteins and the extracellular matrix[126]. It was once considered that due to the affinity for cell surface glycans and extracellular matrix components that CFH was found anchored to these surfaces/structures, and therefore immediately available for regulation of alterative pathway activation. However, it is now generally accepted that the high affinity binding to C3b is a way of recruiting CFH to the cell surface when required, and multiple sites of electronegative attraction (particularly in SCR 20) help to anchor the CFH chain into position to function as a regulator[125]. The net effect of CFH binding to C3b is the rapid disruption and inactivation of the complement cascade. As previously described, this appears to be accelerated by the action of TMBD/C3b binding at the cell surface[123].

Mutations in CFH have been described in multiple studies, and are found in ~30% of patients[16]. The majority of mutations associated with aHUS are heterozygous missense, and do not affect the circulating levels of CFH. Considering the assumed structural arrangement of CFH in fluid-phase (as a 20 SCR chain, without tertiary structure), point mutations appear to only exert localised minor effect[132]. However, heterozygosity means functional effects will be experienced in 50% of the circulating CFH. Interestingly, the observed mutations appear to cluster to the C-terminal end of CFH (**Figure 1.5**), suggesting an issue with binding to cell and extracellular matrix surfaces as a key component to the complement-mediated pathogenesis of aHUS.

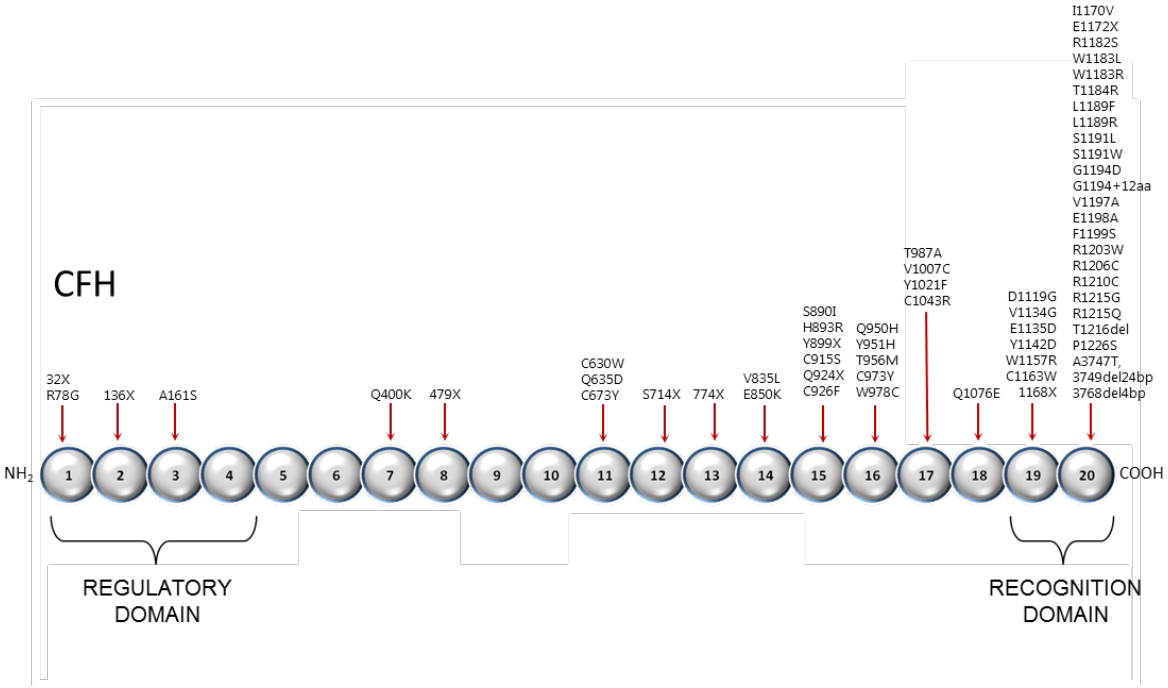


Figure 1.5: Mutations in CFH cluster to the C-terminus in aHUS patients

CFH contain 20 SCR domains which are arranged in a chain. The regulatory domain lies at the N-terminus, and is responsible for decay-accelerator and cofactor activity against the AP C3 convertase. The recognition domain is located at the C-terminus, and is responsible for CFH binding to polyanionic residues (e.g. heparin sulphate) at the cell surface. In aHUS, at-risk mutations cluster to the CFH C-terminus recognition domain. Therefore, abnormalities in the recognition domain are associated with an increased risk of developing aHUS.

Subsequent to these findings, *in vitro* assays using mutant proteins showed no change in fluid-phase complement regulatory activity. However, studies using protein crystallography and nuclear magnetic resonance (NMR) showed that mutations in SCR 20 do have a negative effect on GAG (used as a surrogate for cell surface interaction) binding[125]. Therefore, aHUS-associated mutations in the C-terminus of CFH result in impaired cell surface binding. This leads to dysregulation of complement on host cells. Additionally, CFH has also been shown to bind to the cell surface of platelets *via* interaction with thrombospodin-1, and this interaction in reduced to a significant degree in CFH with C-terminal mutants[133].

Further evidence for the consequences of C-terminal clustering was demonstrated in a mouse model of aHUS. Mice were made transgenic for a truncated version of the murine *Cfh* gene, which lacked exons 19-23, resulting in a protein lacking SCR domains 16-20 (*CfhΔSCR16-20*). Without this surface recognition domain, the mice developed a renal thrombotic microangiopathy analogous to aHUS. There was also evidence of erythrocyte fragmentation, disruption to the endothelium and C3 deposition along the capillary endothelium, arterial smooth muscle and mesangium. Additionally, the endothelium was coated with platelets[134]. However, the *CfhΔSCR16-20* mice were still able to regulate fluid-phase AP activation, as indicated by normal levels of C3 *in vivo*. In contrast, mice with complete CFH deficiency (*Cfh-/-*) develop a renal disease similar to MPGN2, characterised by very low levels of C3, extensive C3 deposition in the glomerulus and dense deposits at the glomerular basement membrane (GBM)[134].

CFH deficiency is also associated with AMD and MPGN2. In particular, at-risk genetic elements have been shown to correlate with each disease. Studies in AMD have shown common *CFH* haplotypes to be associated with disease[135-138]. In particular, a common polymorphism that results in an amino acid change (Y402H) in SCR 7 of CFH and CFHL-1, is found to segregate with disease. In a recent study, we were able to show that the Y402H variant is in some way protective against the development of a CFH autoantibody response in AMD patients (compared to age-matched normal controls)[139]. As with aHUS, other complement genes have also been linked as predisposing factors in AMD[140-145].

In AMD, dense deposits, or ‘drusen’, are found deposited along the Bruch’s membrane in the macular region of the eye, resulting in gradual loss of central vision. These dense deposits have been shown to contain C3 breakdown products, suggesting dysregulation of the AP.

Similar complement containing dense deposits are also found in MPGN2, or dense deposit disease (DDD) as it is also known. Dense deposits were first identified in the GBM, as resolved by electron microscopy. However, in recent studies deposits have also been detected along the Bruch’s membrane, as seen in AMD. Interestingly, MPGN2 is also associated with CFH deficiency, as previous discussed. *CFH* mutations primarily cluster to the N-terminal region of the protein, thus affecting CFH regulatory function (i.e. decay-accelerator and cofactor activity). Therefore, aHUS is associated with deficiency of cell surface recognition, thus affects regulation of AP activation. However, MPGN2 is associated with compromised cell surface and fluid-phase AP complement activation.

Other than deficiency in complement regulatory components, ~80% of MPGN2 patients test positive for C3 nephritic factor (C3NeF), an autoantibody that targets and stabilises the AP C3 convertase. This results in unregulated AP complement activation and consumption of C3 (low C3 is a diagnostic marker of MPGN2). Complement dysregulation in MPGN2 is progressive and destructive, so spontaneous remission is rare. Most patients will progress to end-stage renal failure, with impaired visual acuity[146].

The CFH-associated deficiencies in aHUS, MPGN2 and AMD not only suggest genotype similarities between these diseases, but also a phenotypical relationship.

1.6 The role of CFHR proteins in health and disease

Downstream of CFH in the RCA gene cluster are a group of genes with a high degree of sequence homology to CFH (**Figure 1.6**). These are referred to as the CFH-related (CFHR) genes. The sequence homology between CFH and the CFHR genes is thought to be a result of genomic rearrangements occurring within this region, potentially stemming from a single progenitor gene sequence. Therefore, rearrangements of the progenitor gene sequence eventually, through

a period of evolution, resulted in the birth of a family of genes coding for the CFH and the CFHR proteins. Clearly, the evolutionary selection of this final repertoire of CFH family proteins suggests each family member has a significant role (or multiple roles) to play in the maintenance and survival of the species.

There are five *CFHR* genes (*CFHR1-5*), which result in six mature circulating proteins, CFHR1, CFHR2, CFHR3 CFHR4A, CFHR4B (a truncated CFHR4 splice-variant), and CFHR5. Functional analysis has demonstrated that all CFHR proteins, (except CFHR2) exhibit varying degrees C3b and GAG binding. Whether this is biologically significant is being investigated for each protein.

1.6.1 CFHR1

CFHR1 comprises 330 amino acids, including an 18 amino acid signal peptide. The mature protein is composed of 5 SCR domains and is post-translationally modified with N-linked glycosylation. In human sera, there are two CFHR1 isoforms, CFHR1α (37 kDa) which is non-glycosylated, and CFHR1β (42 kDa) which is glycosylated.

CFHR1 was first discovered when two antigenically related proteins were identified on screening human serum with a CFH pAb[147]. The proteins were purified directly from human sera, and the amino acid sequences of the two were found to be identical. This suggested both proteins were translated from a single mRNA transcript. After deglycosylation with N-glycosidase F, both bands migrated into a single band, suggesting the molecular weight difference was due to N-linked glycosylation. Decay-acceleration was investigated, and cofactor activity assessed using a C3b and CFI is fluid-phase cofactor assay. However, CFHR1 exhibited no evidence of decay-acceleration or cofactor activity.

Subsequent to the identification of this CFH-related protein in human serum, a single cDNA transcript was identified which was directly related to the previously reported protein[148-151]. When nomenclature for the CFH proteins was decided, this protein became known as FHR-1[152], which was later changed to CFHR1.

The functional significance of CFHR1 remained unclear for several years after its initial discovery. However, a recent study demonstrated that CFHR1 is a potent C5 convertase inhibitor[153] and can thus be classified as a complement regulatory protein. Besides acting as a regulatory protein, CFHR1 also plays a role in the defence against infection. It has been shown that both CFH and CFHR1 can associate with neutrophils *via* complement receptor 3 (CR3) to mediate attachment to *Candida albicans* and enhances neutrophil antimicrobial activity[154]. Additionally, CFHR1 has been shown to bind to cell surface proteins (BbCRASP3-5) of *Borrelia burgdorferi*, therefore reducing bacterial virulence[155].

1.6.2 CFHR2

Shortly after the discovery of CFHR1, CFHR2 was identified using the same methodology[156]. CFHR2 has four SCR domains and like CFHR1, exists in two isoforms; non-glycosylated (24 kDa) and glycosylated (29 kDa). There is no evidence to suggest CFHR2 has any complement regulatory activity, and very little is known about potential ligands. However, like other CFHR proteins, it is found associated with lipoproteins in circulation. The CFHR2 gene was found to be highly homologous to the adjacent gene coding for coagulation factor XIIIb[157], suggesting a further link between complement and the coagulation cascade. Dual roles have already been identified with TMBD, as a regulator of thrombin in the coagulation cascade, and C3b in AP complement activation (**Section 1.5.1.5**). Sequence homology between CFHR2 and XIIIb also provides further evidence for evolution of the RCA gene cluster *via* intragenic recombination events, resulting in shuffling of SCR encoded elements.

1.6.3 CFHR3

After previously identifying three mRNA transcripts with significant levels of sequence homology to CFH, cDNA probes were created to search for further gene expression of other potential CFHR transcripts. Another transcript was found, which resulted in the discovery of CFHR3[158].

CFHR3 contains 5 SCR domains, each with respective homology to SCR domains -6, -7, -8, -19 and -20 in CFH. When human plasma is probed *via* western blot, CFHR3 appears in multiple glycosylated isoforms[159].

The functional characteristics of CFHR3 have been investigated, but despite C3b, C3d and heparin binding capacity, the protein is unable to regulate complement activation[160]. Despite this finding, the CFHR3/1 gene deletion has been implicated as a risk factor in aHUS, correlating with the presentation of a CFH autoantibody response[161-164].

1.6.4 CFHR4

CFHR4 protein was first identified in association with triglyceride-rich lipoproteins[165]. The 86 kDa protein was isolated, digested into 6 fragments and the amino acid sequence determined for each fragment. All fragments shared variable levels of sequence homology to CFH, CFHR1, CFHR2 and CFHR3. In parallel the cDNA transcript for CFHR4 was also identified, and matched to the amino acid sequence.

Functional characterisation showed CFHR4 binds to C3b *via* the C-terminus in a dose-dependent manner, suggesting it may have complement regulatory activity at the level of C3 convertase[160,166]. However, like all CFHR proteins, CFHR4 lacks the complement regulatory domain found in CFH (SCR domains 1-4). On an SDS-PAGE separation, bands of 43 kDa and 86 kDa were seen, the latter of which was considered a CFHR4 homodimer.

To assess the function of CFHR4, the protein was expressed recombinantly in a baculovirus/insect cell system[160]. Recombinant expression led, as *in vivo*, to both the monomeric and homodimeric forms of CFHR4. CFHR4 was shown to be post-translationally glycosylated, and also to bind C3b. It was later determined that CFHR4 was a splice-variant of a larger cDNA transcript. In serum, the size of the larger CFHR4 protein is 86 kDa, suggesting the previously reported CFHR4 homodimer may actually have been this larger protein[167]. The two proteins became respectively known as CFHR4A (86 kDa) and CFHR4B (45 kDa).

A recent study examined the functional significance of the C3b binding capability of CFHR4 because it is unable to regulate C3 convertase formation. CFHR4 was shown to bind C3b at the cell surface and reduce the decay accelerating activity of CFH[168]. This suggested a novel role for CFHR4 in promoting rather than regulating complement activation.

1.6.5 CFHR5

CFHR5 (65 kDa) was the final CFHR protein to be discovered, as reflected by its nomenclature. The protein was first identified in the analysis of complement deposits in the glomerulus[169,170]. A mAb was raised against CFHR5 using pathological human glomerular preparations as the immunogen. CFHR5 was subsequently purified *via* affinity chromatography and shown to bind C3b *in vitro*. The CFHR5 gene was mapped to the RCA gene cluster and is located between the genes encoding CFHR2 and Factor XIIIb[171]. CFHR5 was eventually shown to bind heparin, have cofactor activity, inhibit C3 convertase and bind CRP and lipoproteins[172]. This suggests that CFHR5 may have multiple roles in complement regulation/activation. Genetic variants of CFHR5 have been associated with aHUS[173], MPGN2[174] and the recently characterised CFHR5 nephropathy[175].

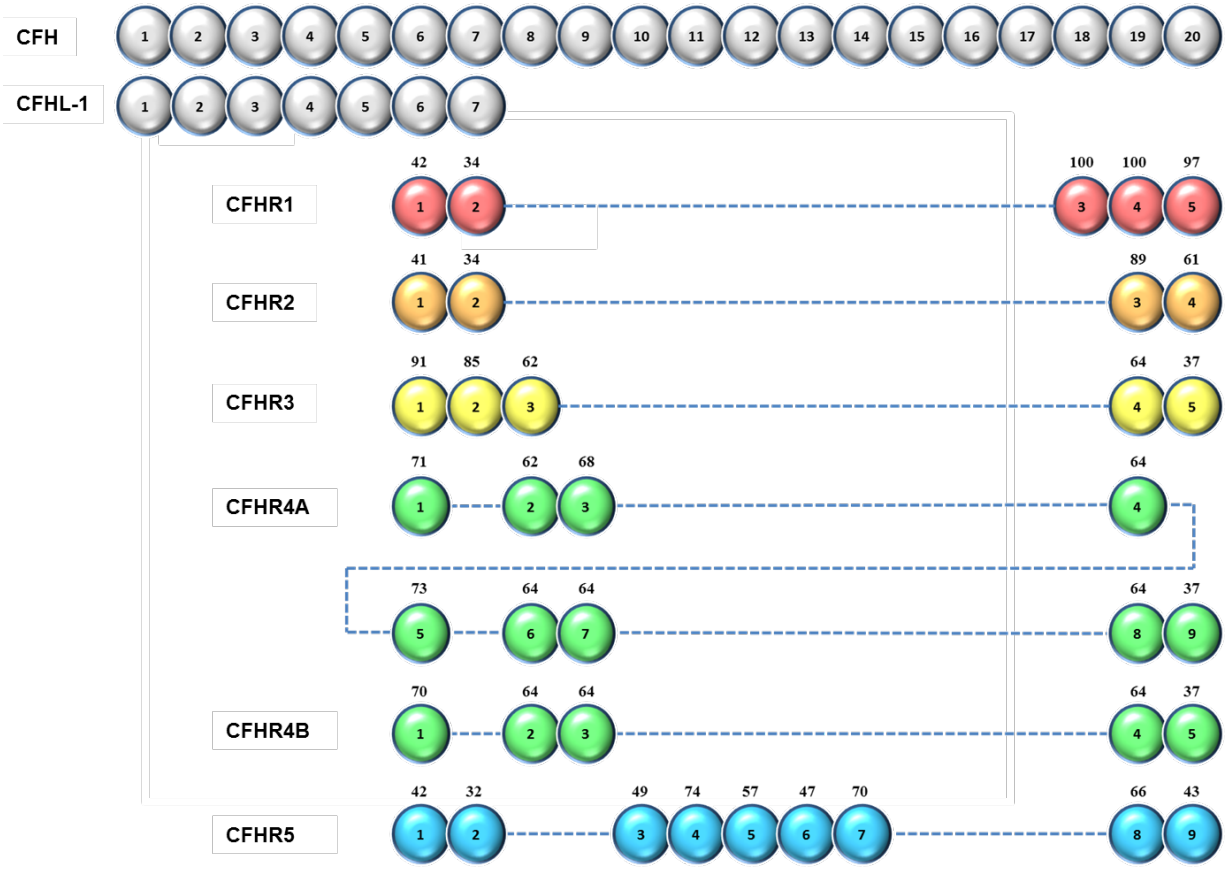


Figure 1.6: Comparison of sequence homology in CFH family proteins

CFH and the CFHR proteins share a high degree of sequence homology. The SCR domains of the CFHR proteins are aligned according to their homology to CFH SCR domains 1-20. Above each CFHR SCR domain is the % sequence homology to the corresponding CFH SCR domain located above.

1.6.6 NAHR within the RCA gene cluster

The RCA gene cluster is scattered with regions of sequence homology that span the CFH family of genes. Under normal conditions, regions of sequence homology line-up (allele to allele), and recombination events occur leading to an alternative arrangement of haplotypes (alleles) within the population. However, within the RCA gene cluster, the region is scattered with repeats of sequence homology. This has the potential to result in regions of sequence homology lining-up in a non-allelic fashion. This can allow recombination to occur that results in an alternative arrangement of genes on each chromosome. This process is known as non-allelic homologous recombination (NAHR). When NAHR occurs in the non-coding regions spanning genes, one chromosome loses genes, and the other chromosome gains them, leading to an increase in gene copy number (**Figure 1.7**). When NAHR occurs within the coding sequence, the resulting gene product will either be a truncated gene/protein or create a novel fusion gene/protein[176,177]. Within the normal population, CFHR copy number variation is common with CFHR3, CFHR1 and CFHR4. However, due to the location of non-allelic regions of sequence homology, CFHR3 and CFHR1 are found deleted together in a single block. Based upon the theoretical understanding of how CFHR3/1 is deleted, there should also be an equal frequency of chromosomes with two copies of CFHR3 and CFHR1. In aHUS patients, ~10% have a homozygous deletion of CFHR3/1. This implicates both CFHR3 and CFHR1 as risk factors in the presentation of aHUS. It soon became clear the CFHR3/1 deletion correlated with the presentation of an IgG CFH autoantibody response in ~90% of these CFHR3/1 deficient patients[162,163,178]. After several reports of the CFHR3/1 deletion and CFH autoantibody phenotype, Zipfel *et al*., (2010) proposed these patients as representative of a novel aHUS subtype, referred to as ‘Deficiency of CFHR plasma proteins and Autoantibody Positive form of HUS’, or DEAP-HUS[179].

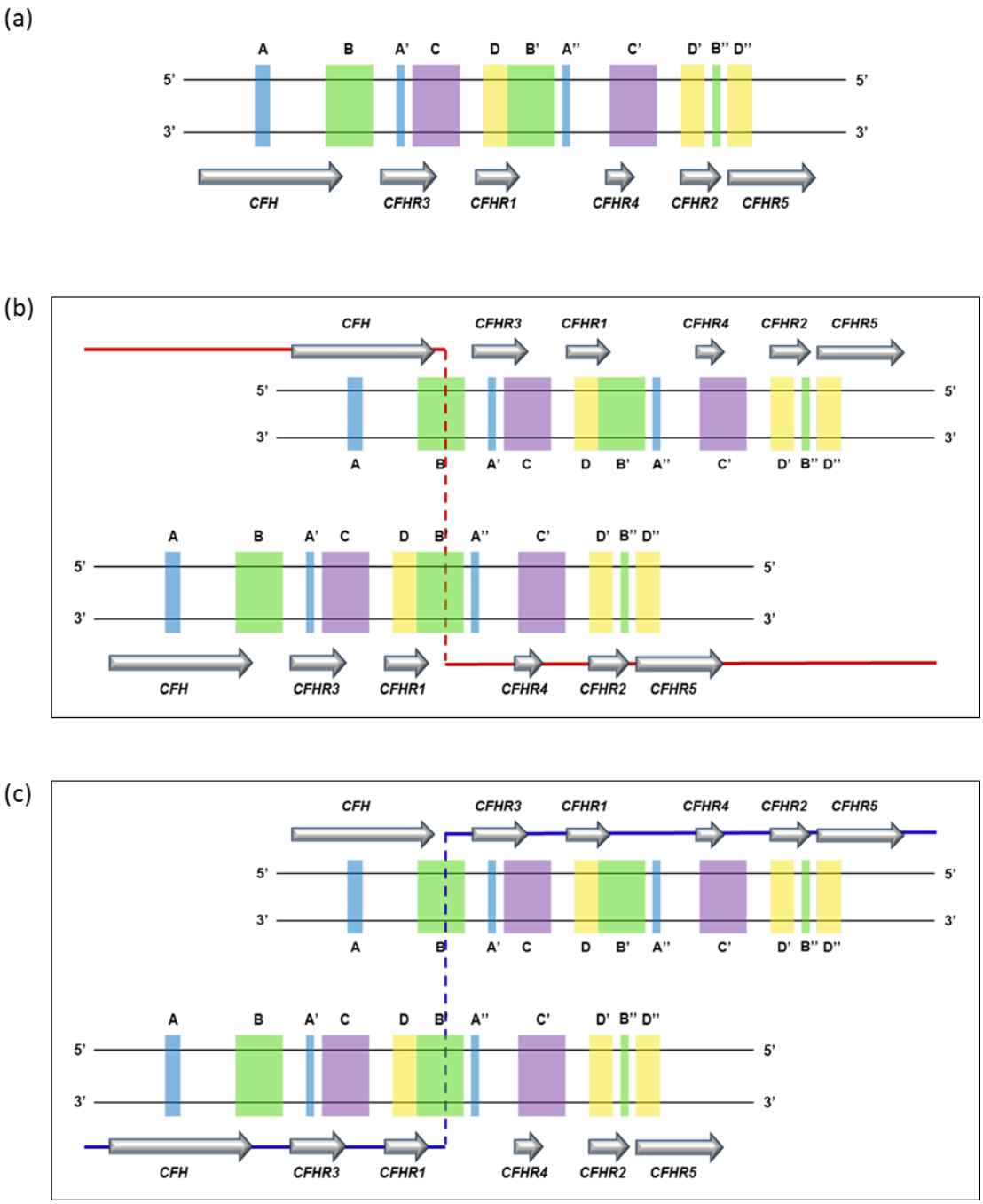


Figure 1.7: NAHR in the RCA gene cluster

(a) The *CFH* genes are arranged in tandem (grey arrows), and regions of high DNA sequence homology are highlighted in colour, and labelled A-D. Additional labelling with ' or '' indicates the position of each segment of sequence homology. **(b)** B shares sequence homology with B' so they align and NAHR occurs. The red line indicates which genes will be carried forward on the first chromosome. NAHR has resulted in a deletion of CFHR3 and CFHR1 in one block. **(c)** The blue line demonstrates what genes will be carried forward onto the second chromosome. NAHR has resulted in the duplication of CFHR3 and CFHR1, essentially taken from the first chromosome.

That the CFHR3/1 deletion is associated with CFH autoantibodies suggests that CFHR proteins may have a role to play in maintaining immunological tolerance to CFH. Therefore, measuring CFHR plasma concentration may be a disease marker for an autoimmune subtype of aHUS. A common methodology for measuring plasma protein concentration is the use of sandwich ELISA, where the sensitivity of detection can reach pg/ml. While there are commercially available kits for measuring plasma CFH concentration, there are no such kits for CFHR1, CFHR3 or CFHR4. Additionally there were no CFHR1-specifc (no cross-reactivity with other CFH family proteins) mAbs available at the start of this study. Commercially available CFH mAbs/polyclonal Abs (pAbs) inevitably cross-react with the CFHR proteins due to the high levels of sequence homology between the proteins. From the outset of this research project, there were two commercially available CFH mAbs which bound to CFH SCR 20 (C18/3 and L20/3; Santa Cruz Biotech). As CFH SCR 20 has >97% amino acid sequence homology with CFHR1 SCR domain 5, both Abs cross-react with CFHR1. These Abs could therefore not be used to specifically measure the concentration of either CFH or CFHR1. Additionally, there were no mAbs available at the start of this research against CFHR3 or CFHR4. Therefore, there was a need for the production of a panel of CFHR-specific mAbs.

Having mAbs targeting different regions of the CFHR proteins would also be useful in the investigation of patients in whom genetic analysis identifies copy number variants in the CFH family of genes. Having a repertoire of Abs to target particular regions of interest is a powerful research tool.

1.7 Complement and Autoantibodies

In MPGN2 autoantibodies have been described against CFH, CFB[180], and more importantly, against C3 convertase. Approximately 80% of MPGN2 patients present with C3NeF, which functions to stabilise C3 convertase into an activated state[181,182]. The result is continuously unregulated activation of the C3 amplification loop. The origin of this Ab response is still unknown.

In a recent study from our research laboratory, a cohort of AMD patients was tested for CFH autoantibodies. These were compared to a group of age-matched controls. The results demonstrated that AMD patients with a CFHR3/1 deletion, had a lower level of CFH autoantibodies, compared to the control. This suggested the CFHR3/1 deletion was in some way protective against forming a CFH autoantibody response. Additionally, it highlighted that developing CFH autoantibodies may be related to old age.

In aHUS, autoantibodies have been described targeting CFH and CFI. As previously described, CFH autoantibodies were discovered in the plasma of 90% aHUS patients with homozygous deficiency of CFHR3/1. In the remaining 10% with the deletion, there was no evidence of CFH autoantibodies. When investigating an autoantibody response, it is important to consider that Ab titres are going to be variable in any one individual, based upon the proposed requirement for the immunological response (i.e. the antigen availability with the immune-sensitised individual). As CFH is consistently available, it seems paradoxical that the CFH autoantibody response would not be switched on continuously. Instead, aHUS patients have shown variability in their autoantibody titres over time. Transplantation and pregnancy are key triggering events for the activation of increased levels of CFH autoantibodies.

To date the functional consequences of having CFH autoantibodies remains unclear. Certainly, cell surface blockade has been shown *in vitro* and treatment of patients with CFH autoantibodies with B-cell depleting therapy has been advocated by several reports. It is not clear whether autoantibodies can induce disease on their own by either directly blocking CFH function or forming immune complex in the kidney. Therefore, understanding any similarities to other immune complex kidney diseases may elucidate this. IgA Nephropathy (IgAN)

is a disease where significant immune complex deposition is seen and it would be interesting to determine whether autoantibodies to CFHR proteins are present in this disease. Furthermore, CFHR5 nephropathy has demonstrated that CFHR5 may be an important complement regulator in the glomerulus. In aHUS, CFH deficiency from genetic mutation causes, or contributes towards the onset of disease. This is also suggested by the IgG autoantibody response to CFH. In IgAN, Ab mediated autoimmunity is present so there may be other autoantibody-mediated events occurring with regards to complement activation (i.e. targeting complement regulatory proteins, like CFHR5). Should CFHR5 autoantibodies be discovered, this would provide further evidence for CFHR5 as a major complement regulator in the kidney.

1.8 aHUS: a multiple hit disease

There are a range of potential risk-factors involved in the pathogenesis of aHUS. Based upon the predisposing factors (as previously presented), these can be summarised as rare genetic variants (mutations or gene rearrangements), common genetic variants (SNPs), and complement-targeted autoantibodies. The onset of disease appears to require several of these risk factors, in combination with a trigger, such as infection, pregnancy or transplantation. This has been described as a 'multiple-hit' theory[183] (**Figure 1.8**).

When considering 'a multiple hit' hypothesis, it is important to consider this in relation to all diseases associated with complement dysregulation. Additionally, all complement proteins involved in the AP must be considered. In a recent study investigating high risk SNPs in AMD, it was demonstrated that a combination of common polymorphisms in *C3*, *CFB* and *CFH* (from one individual) was associated with the onset of disease[184]. Heuric *et al*., tested the functionality of each polymorphism and confirmed that each risk-associated SNP did result in reduced complement regulation. When combining all 3 risk-associated proteins in a haemolytic assay, haemolysis was increased 6-fold providing evidence that the risk-associated polymorphisms were having a negative effect on AP complement regulation. They referred to this as a high-risk 'complotype' (or combination of polymorphisms).

A similar mechanism has been described within individual genes, whereby groups of risk-associated polymorphisms can be inherited together, resulting in risk-associated haplotypes. These have been shown in aHUS, AMD and MPGN2[134].

It was recently reported that a female post-transplant patient had developed postpartum aHUS, five years after receiving a donor liver. After screening several complement genes in both the donor and recipient, it was discovered that the donor liver was heterozygous for a *CFH* nonsense mutation leading to a premature stop codon in SCR 6. The recipient had a homozygous *CD46* at-risk haplotype (termed $CD46_{GGAAC}$). This combination of donor/recipient genes (or complotype) did not result in the presentation of aHUS. However, in this case pregnancy appears to have served to trigger aHUS, in combination with the native and acquired aHUS risk alleles. This is prime example of multiple hits resulting in the onset of aHUS.

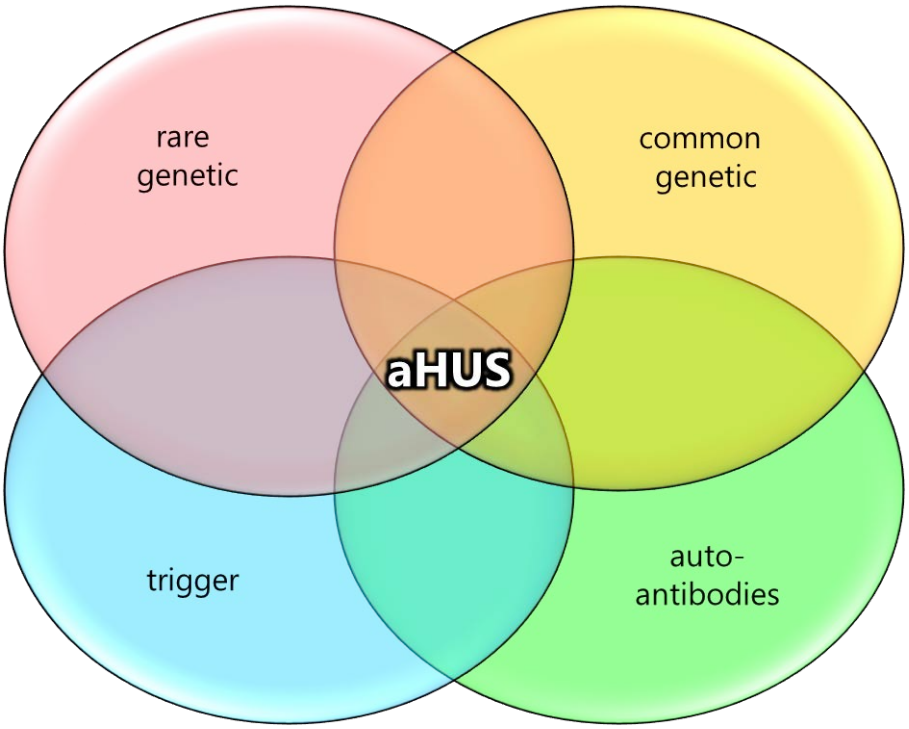


Figure 1.8: Multiple risk factors are required to develop aHUS

In aHUS it has been proposed that multiple risk factors within AP complement components are required to trigger the onset of disease. This hypothesis is represented diagrammatically above, demonstrating that when risk-factors overlap aHUS is likely to ensue. Disease predisposing risk factors have been grouped into rare genetic abnormalities (e.g. point mutations, gene rearrangements), common genetic features (e.g. SNPs, haplotype variants), autoantibody presentation (e.g. targeting CFH and CFI) and trigger incidents (e.g. pregnancy, infection, transplantation).

1.9 Aims of this research project

The primary aim of this research project is to produce a set of recombinant human CFHR proteins, to be utilised in the investigation of CFHR involvement in the disease pathogenesis of autoimmune aHUS and IgAN.

After producing the proteins, the aim is to establish a set of quantification assays to measure CFHR levels in human plasma. CFHR1, CFHR3 and CFHR4 deficiency are linked to the production of CFH autoantibodies. However, deficiency is measured first by assessing gene copy number, followed by analysis of protein in plasma. While this methodology has proved useful, it has not enabled us to conclude on which of these CFHR deficiencies is the triggering element. CFHR1 is the common deficiency between the two block deletions (*CFHR3/1* or *CFHR1/4*), so it is assumed to be the triggering element. However, further clarification is required. Additionally, plasma CFHR deficiency may result from issues other than gene deletions, like promoter or regulatory element mutations. Therefore, screening CFHR levels at the level of secretion is more representative.

Due to the lack of suitable commercially available mAbs, the second major aim of this thesis is to produce a set of CFHR-specific mAbs, for use in the development of the quantification assays. Therefore, the recombinant CFHR proteins will be utilised to illicit an Ab response in mice, followed by the production of CFHR-specific Ab-secreting hybridomas.

The final aim of this thesis is to establish the autoantibody status of a cohort of aHUS and IgAN patients, using the recombinant CFHR proteins as candidate coating antigens in optimised ELISA screens.

Chapter 2

2. Materials and Methods

2.1 DNA Manipulation and Cloning

2.1.1 DNA plasmids and primers

The DNA expression vector *pDEF(nMCS)* (**Figure 2.1**) has been commonly used in conjunction with CHO cells to produce a range of human proteins, including human complement proteins[185-188]. The vector contains an *elongation factor 1α* (*eF1α*) promoter region 5' of the multiple cloning site (MCS) which results in constitutive protein expression when transfected into a mammalian cell system. The vector also contains the hygromycin B resistance gene (*hyg*)[189,190], which allows selection of transfected cells using hygromycin B supplemented media. This was chosen as our base construct, but modified to include a 6x poly-histidine motif (HisTag).

Single stranded oligonucleotide primers were designed to produce a clone of each *rCFHR* cDNA (**Table 2.1**). For *CFHR1*, *CFHR2*, *CFHR3*, and *CFHR4B*, *XbaI* and *BamHI* restriction endonuclease sites were selected/introduced as the most suitable for insertion of each clone into *pDEF/L5H6*. Due to an internal *XbaI* site in the *CFHR5* sequence, an alternative RE site was selected for the *CFHR5* forward primer. Therefore, *SpeI* and *BamHI* RE sites were selected for cloning of *CFHR5*. Primers were synthesised by Eurofins MWG Operon (Eisberg, Germany).

PCR Template	5'>3' Forward Primer (FP)	5'>3 Reverse Primer (RP)
CFHR1	*CCAAGCTCTAGAATGTGGCTCCTGGTCAGTGTAATT*	*TTGATTGGATCCTCTTTTTGCACAAGTTGGATACTC*
CFHR2	*CFHR1 FP used*	TTGATTGGATCCTTTTTCTTCACAACTGGGATATAC
CFHR3	TCTAACTCTAGAATGTTGTTACTAATCAATGTCATT	GCTGCCGGATCCTTCGCATCTGGGGTATTCCACTAT
CFHR4	*CFHR3 FP used*	CGACGGGGATCCTTCGCATCTGGGGTATTCCACTAT
CFHR5	CCAAGCACTAGTATGTTGCTCTTATTCAGTGTAATC	TGTGAAGGATCCTTCACATATAGGATATTCAAATTTCCC
pDEF(MCS)	CTCAAGCCTCAGACAGTGGTGC	CTGCATTCTAGTTGTGGTTTGTCC

Table 2.1: CFHR primers

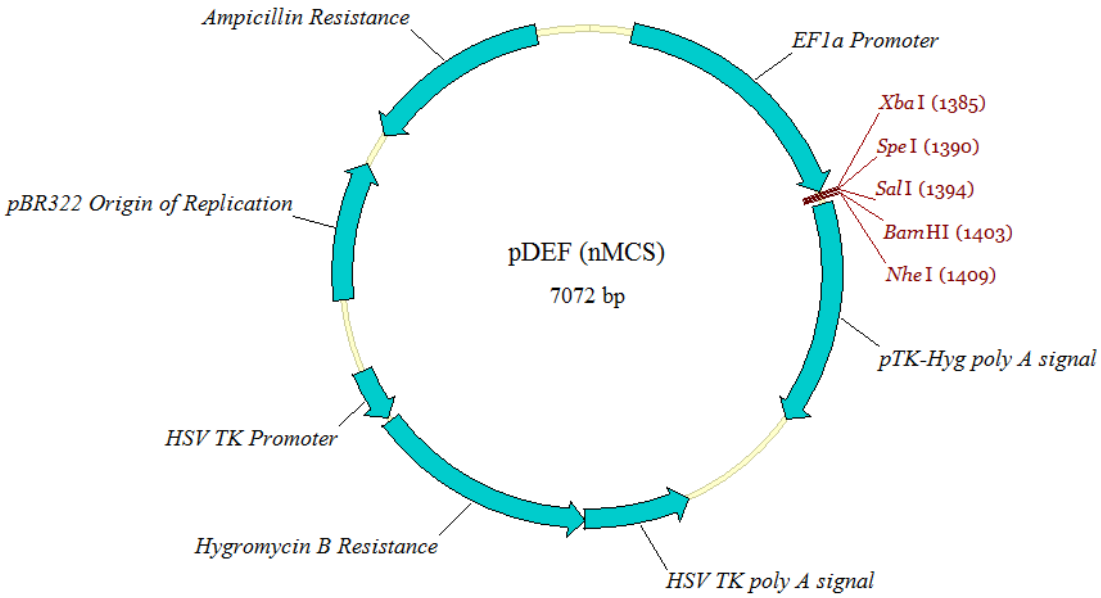


Figure 2.1: An illustration of the expression vector *pDEF/nMCS*

The *pDEF/nMCS* cloning vector contains multiple features which are important for plasmid selection and replication in bacteria, and gene transcription and protein expression in mammalian cells.

2.1.2 DNA Sequencing

Complementary forward (*pDEF_NF*) and reverse (*pDEF_NR*) primers were designed to anneal upstream (1326-1346bp) and downstream (1495-1518bp) of the MCS. DNA was sequenced by Eurofins MWG Operon (Eisberg, Germany).

2.1.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify DNA in the cloning of *CFHR* cDNA, and also in the screening of transformation colonies *via* 'Colony PCR'.

2.1.3.1 *CFHR* cDNA PCR Amplification

CFHR cDNA was obtained from multiple sources (**Table 2.2**), and used as the template in a standard PCR recipe (used for all *CFHR* cloning,**Table 2.3**). *Pfu* DNA polymerase (Fermentas, UK) was used for amplification as it possesses 3'-5' proof-reading activity. Using *Taq* DNA polymerase would result in a 6-fold increase in the number of mutations introduced per duplicated base pair, when compared to the mutation rate of *Pfu.* This reduces the frequency of point mutations introduced into each *CFHR* cDNA sequence. *CFHR*-specific primers were used, as previously described (**Section 2.1.1**). PCR condition are summarised below (**Table 2.4**). All PCR results were assessed on 1% agarose gels, supplemented with 0.5 µg/ml ethidium bromide. UV emissions were visualised using the AlphaImager® Gel Documentation system (ProteinSimple; Santa Clara, USA). After PCR, amplified products were purified using a 'QIAquick PCR Purification Kit' (QIAGEN, UK), following the manufacturer's instructions.

cDNA Clone	Storage Vector	Antibiotic Resistance	Vector/cDNA Source
CFHR1	*pDNR-LIB*	Chloramphenicol	IMAGE Consortium
CFHR2	*pUC-19*	Ampicillin	Synthesised by 'Genscript'
CFHR3	*pDNR LIB*	Chloramphenicol	IMAGE Consortium
CFHR4B	*pCR4-Topo*	Kanamycin	IMAGE Consortium
CFHR5	*pGEM-easy*	Ampicillin	M. Pickering; Imperial College London, UK

Table 2.2: Sources of CFHR cDNA

Recipe	Volume
Template (1ng/µl)	1µl
Forward Primer (50µM)	1µl
Reverse Primer (50µM)	1µl
dNTP mix (25mM each)	1µl
10x *Pfu* Buffer with $MgSO_4$	5µl
Pfu DNA polymerase (2.5u/µl)	1µl
dH_2O	40µl
Final Volume:	**50µl**

Table 2.3: Standard PCR Recipe

Recipe	Volume
Bacterial Lysate*	5µl
Forward Primer (50µM)	1µl
Reverse Primer (50µM)	1µl
dNTP mix (25mM each)	1µl
10x Thermo Buffer	5µl
Taq DNA polymerase (2.5u/µl)	1µl
dH_2O	35µl
Final Volume:	**50µl**

Table 2.4: Colony PCR Recipe

Step	Temperature	Duration	Cycles
Melting	95°C	5 mins	1
Melting	95°C	1 min	35
Annealing	55°C	1 min	
Extension	72°C	*Taq*: 1 min/kb *Pfu*: 1.5 min/kb	
Extension	72°C	7 mins	1
Storage	4°C	∞	-

Table 2.5: Standard and Colony PCR Thermo Cycler Conditions

2.1.3.2 Colony PCR Amplification

After DNA plasmid transformation into bacterial cells, ampicillin-resistant colonies were selected and screened for the presence of the correct *CFHR* insert.

Bacterial colonies were picked using a sterile filter-tip, and resuspended in 20 µl dH_2O and boiled at 100°C for 5 min. The lysed samples were micro-centrifuged for 5 min at 13,000 x g, to pellet the cell debris from the sample lysate. The supernatant (SN) was collected, and 5 µl used as template DNA in a colony PCR (**Table 2.5**). *pDEF_NF* and *pDEF_NR* primers were used to amplify the contents of the *pDEF/L5H6* MCS.

As colony PCR amplification products were not used after screening, a proof-reading polymerase was not required. Therefore, *Taq* DNA polymerase (New England Biosciences, UK) was used in colony PCR reactions.

2.1.4 DNA Digestion & Ligation

To produce a *CFHR* expression construct, PCR amplified *CFHR* cDNA was RE digested and inserted into the *pDEF/L5H6* expression plasmid. The PCR product will be referred to as the 'insert' and the expression plasmid as 'vector'.

The insert and vector were double-digested using *XbaI*/*BamHI* (*CFHR1-4B*), or *SpeI*/*BamHI* (*CFHR5*), following the manufacturer's instructions (New England Biosciences, UK) for double digestions. Sufficient insert and vector DNA was used so that 100% would digested within 1-2 hr, incubating in a thermocycler at 37°C. Following digestion, insert and vector DNA were purified using a 'QIAquick PCR Purification Kit' (QIAGEN, UK).

Purified insert and vector DNA concentrations were determined *via* spectrophotometry at 260 nm, and a 1:1 molar ratio of each was used for the ligation reaction. T4 DNA ligase (Fermentas) was used in conjunction with the manufacturer's guidelines

2.1.5 Bacterial Strains

2.1.6 Plasmid Transformation and Purification

'OneShot® Top10' *E.coli* were used for DNA transformation using the manufacturer's 'heat-shock' protocol (Invitrogen, UK). Transformed bacterial cells were selected using Luria-Bertani (LB) broth or agar, supplemented with 50 µg/ml ampicillin. Plasmids were purified from bacterial cells using a 'QIAprep Miniprep Kit' (QIAGEN, UK) following the manufacturer's instructions. Plasmids were stored frozen at -20°C, until required.

2.2 Tissue Culture

2.2.1 Cells, Media and Maintenance

Chinese Hamster Ovary (CHO) cells were the selected cell line for the expression of rCFHR proteins. The cell line had previously been used in-conjunction with the *pDEF(nMCS)* vector for expression of complement proteins. CHO cells were maintained in Ham's F12 media, supplement with 10% FCS, 2 mM L-glutamine, 0.1 Units/ml penicillin and 100 µg/ml streptomycin (PAA, UK). CHO and all other mammalian cell lines used were incubated at 37°C, 5% CO_2, with humidity.

CHO cells were split once they reached 70-80% confluence on the flask base. Being an adherent cell line, trypsin-EDTA was used detach cells from the plastic followed by splitting cells 1/10.

NS0 cells were selected as the fusion-partner cell line (**Section 1.3.3**) for production of immortalised hybridomas. NS0 cells were cultured in RMPI media supplemented with 10% IgG stripped FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM amino acids (1/100 v/v; PAA, UK), 1 mM kanamycin and β-mercaptoethanol (1/1000 v/v).

As NS0 cells were incubated with phenol supplemented RPMI media, the requirement to split cells was monitored by the growth media changing colour from red/pink, to orange/yellow, indicating an increase in the environmental pH. Cells were generally split 1/10 or 1/20, depending on density of cells.

Post-fusion hybridomas cells were cultured using the same media and conditions as the NS0 cells, with the addition of HAT supplement (0.5 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine; Gibco, UK).

2.2.2 Cell Transfection and Selection

2.2.2.1 CHO cell transfection

CHO cells were transfected with *pDEF/rCFHR* plasmid DNA using transfection reagent jetPEI® (Polyplus Transfection, France). The jetPEI® transfection reagent is a polyethelenimine (PEI) reagent with an abundance of protonated nitrogen residues which interact with the negatively charged DNA phosphate backbone to form DNA/jetPEI® complexes[191]. The ratio of jetPEI® to DNA is calculated to confer a net positive charge to the surface of the DNA complexes, resulting in the electrostatic interaction with anionic cell surface proteoglycans. Complexes are then internalised *via* endocytosis, into endosome compartments. Endocytosed material is usually subjected to a low pH environment, resulting in degradation of 'exogenous' material. However, the nitrogen-rich jetPEI® acts as a "proton-sponge", therefore buffering the endosomal compartment and protecting DNA degradation. Disruption of the compartmental pH causes a disruption to the osmotic balance leading to endosome rupture. The subsequent release of DNA into the cell cytoplasm thereby facilitates nuclear transport of DNA into the nucleus for genome integration and transcription.

The transfection of all *pDEF/rCFHR* plasmids was completed using 24-well tissue culture plates. CHO cells were seeded 24 hours prior to transfection, seeding $5*10^4$ trypsinised cells per well (surface area = $1cm^2$) in 0.5 ml volume of CHO cell media.

The *pDEF/rCFHR* plasmid DNA and jetPEI® were individually diluted in 150 mM NaCl (**Table 2.6**). The two solutions were then combined by adding the jetPEI®/NaCl solution to the DNA/NaCl solution, followed by vortexing for 15 seconds and incubating at room temperature (RT) for 30 minutes (min). With complexes now formed, the total volume of the DNA/jetPEI® was added 'drop-wise' to each culture well, homogenising by gently agitating the plate in a

'swirling' motion. Plates were incubated for 24 hr using conditions described for CHO cells.

Further details on nitrogen/phosphate ratios can be referenced in the manufacturer's instructions (CPT 101Vn; last updated March 2012).

Plate	Amount of DNA (µg)	Volume of jetPEI® (µl)	Volume 150mM NaCl per DNA and jetPEI® (µl)	Total volume of complexes added per well (µl)
24-well	1	2	50	100

Table 2.6: Complex preparation from transfection using jetPEI® reagent

Controls were included to monitor the effect of antibiotic selection on wild type verses transfectant CHO cells. These included a no DNA negative control, to monitor cell death when under antibiotic selection pressure. A *pDEF(nMCS)* transfection was also included to act as a positive control for antibiotic selection and a negative control for recombinant protein production. Untransfected CHO tissue culture SN was also used as a negative control in downstream screening of *pDEF/rCFHR* transfected cultures.

Screening of *pDEF/rCFHR1*, *pDEF/rCFHR3*, *pDEF/rCFHR4B* and *pDEF/rCFHR5* transfectants was achieved by sandwich ELISA (**Section 2.3.4.2**), and *pDEF/rCFHR2* by western blot (**Section 2.3.6**).

2.2.2.2 Selection of CHO cell transfectants using hygromycin B

CHO cells transfected with *pDEF/rCFHR* plasmids are under the selective pressure of hygromycin B. However, prior to selection of transfectants, a suitable concentration of hygromycin B was ascertained by running a 'cell death curve'.

CHO cells were seeded to individual wells of a tissue culture plate and incubated for 24 hr in CHO cell media. This media was then exchanged for CHO cell media supplemented with a serial dilution of hygromycin B (1000-0 µg/ml). Cultures were incubated for 10 days (d), with trypsin-EDTA stripping and reseeding of all cells every 2 d. From previous experience, CHO cells appear to maintain some sense of resistance to the detrimental effects of antibiotic pressure if left in their adherent state. This is most likely not resistance to the

antibiotic, but rather maintenance of the adherent morphology due to cell surface protein interaction with the culture vessel surface. Stripping CHO cells under hygromycin B selection conditions appears to be a more effective approach, as the inhibition of protein synthesis prevents CHO cells from reattaching as efficiently to the vessel surface. Therefore, no adherent cells are maintained in the culture.

At each stage of stripping and reseeding (d +2, +4, +6, +8, and +10), cells were counted using a haemocytometer using the manufacturer's instructions (Abcam).

The results suggest using hygromycin B at 200 µg/ml would result in clearance of non-transfected CHO cells within 10 d (**Figure 2.2**). At 400-600 µg/ml this is reduced to approximately 6-8 d. The most efficient concentration is between 800-1000 µg/ml, taking 4 d until CHO viability is no longer present. These results are based upon wildtype CHO cells, but cannot inform on how transfected cells may respond to different concentrations of hygromycin B. Therefore, using a hygromycin B concentration in the lower range will increase the chances of transfectant cell survival, especially as the transfected cells adapt to their transgenic status under the selective pressure of the hygromycin B antibiotic. Therefore, 400 µg/ml was chosen to facilitate transfectant selection within 7 d.

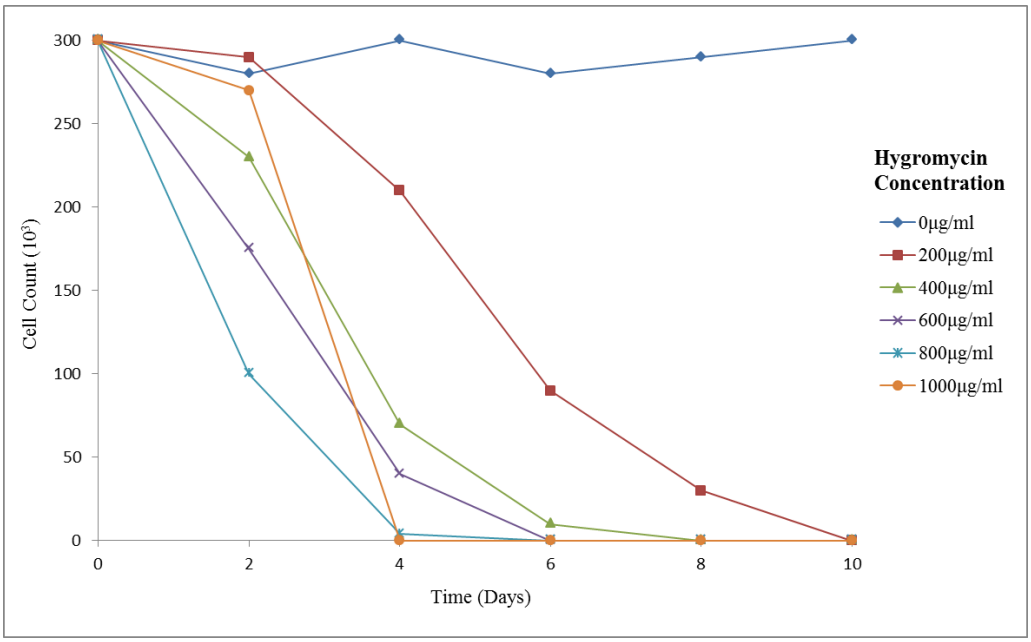


Figure 2.2: CHO cell death curve using hygromycin B

CHO cells were seeded onto plates and treated with an increasing concentration of hygromycin B. Cells were trypsinised every 2 d counted using a haemocytometer. After counting, all cells were returned to the well with fresh CHO cell media supplemented with the relevant concentration of hygromycin B.

2.2.3 Cryogenic Storage of Mammalian Cells

For long-term storage of mammalian cells, cell cultures were pelleted at 300 x g, and resuspended in foetal calf serum (FCS) supplemented with 10% dimethyl-sulphoxide (DMSO). Cells were immediately placed in a polystyrene box and stored overnight at -80°C, to facilitate gradual freezing. Cells were then transferred to liquid nitrogen (LN_2) for long-term storage.

To revive cryogenically frozen mammalian cells, culture vials were rapidly defrosted by incubation in a pre-heated 37°C water bath for 5 min. Defrosted cells were then diluted with pre-warmed sterile phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and pelleted at 300 x g. The SN was discarded, and the pellet resuspended in at least 10 ml of pre-warmed supplemented media (**Section 2.2.2**). Cells were allowed to revive for at least 48 hr before being used in experimental procedures.

2.3 Protein Purification and Identification

Proteins were purified using HiTrap™ Chelating HP, HiTrap™ Protein G HP and HiTrap™ NHS-activated HP columns (GE Healthcare, UK). All buffers, tissue culture SNs and serum solutions were adjusted to pH 7.4 (unless specified otherwise) and filter sterilised using 0.22 µM Corning® Filtration System units (Corning, UK), prior to running over each chromatography column.

When running samples over each column, an AKTAprime purification system was used to regulate flow-rate, concentration gradients and the monitoring of protein elution. AKTAprime was supported by UNICORN version 5.0 software (GE Healthcare, formerly Amersham Biosciences).

After eluting column matrix bound proteins, elution fractions were initially assessed *via* SDS-PAGE and Coomassie staining (**Section 2.3.5**).

2.3.1 HisTag Purification

All rCFHR proteins were designed with a C-terminal HisTag to enable rapid purification *via* a nickel-coated chromatography column. ‘HiTrap™ Chelating HP’ columns (GE Healthcare, UK) were coated with 0.5 ml of 0.1 M nickel

sulphate, as described by the manufacturer's instructions. The column was primed with 'His Binding Buffer' (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole).

CHO cells transfected with *pDEF/rCFHR* expressions plasmids were cultured using the conditions used previously described (**Section 2.2.1**), until 500 ml of culture SN was collected. SN was centrifuged at 4,500 x g for 10 min to remove cell debris, followed by a 1:1 dilution with '2x His Binding Buffer' (40 mM sodium phosphate, 868 mM NaCl, 40 mM imidazole).

The SN solution was passed over the primed nickel-coated 'HiTrap™ Chelating HP' column at a constant flow rate of 1 ml/min. The column was then washed with 'His Binding Buffer' to wash unbound SN protein, prior to the elution step. The column was washed until the protein absorbance trace (A_{280}) had returned to baseline.

The column was then eluted using a concentration gradient from 100% 'His Binding Buffer', to 100% 'His Elution Buffer' (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole), set to run at a flow rate of 1 ml/min for 10 min.

2.3.2 mAb Purification

Monoclonal IgG Abs were purified from hybridoma cultures using 'HiTrap™ Protein G HP' columns (GE Heathcare, UK). Hybridomas were cultured using the conditions previously described (**Section 2.2.1**), until 300 ml of culture SN was collected. SN was centrifuged at 4,500 x g for 10 min to remove cell debris, followed by a 1:1 dilution with PBS and the addition of 0.01% NaN_3.

A 1 ml 'HiTrap™ Protein G HP' column was primed with PBS/NaN_3 0.01%, to prime the column ready for running the SN solution. The diluted SN ran over the column at a rate of 1 ml/min. The column was then washed with 5 ml PBS/NaN_3 0.01% to wash unbound SN protein, prior to the elution step.

The column was eluted using 0.1 M glycine HCl (pH 2.5), collecting 1 ml elution fractions until all bound protein had eluted. To neutralise the acidic elution buffering, 100 µl of 1 M Tris base (pH 11.0) was added to each fraction tube before Ab elution.

2.3.3 Purification of native CFHR proteins

'HiTrap™ NHS-activated HP' columns (GE Heathcare, UK) were covalently coated with R1/1037 or R4/244, following the manufacturer's instructions (GE Healthcare). Human donor serum was collected, and 30 ml was diluted 1/10 with PBS/NaN_3. After running the 300 ml of diluted serum over the column, the column was eluted and analysed as previously described (**Section 2.3.2**).

2.3.4 Enzyme-linked Immunosorbent Assay

2.3.4.1 Direct ELISA

Direct ELISA was used in several methods for the detection of protein 'directly coated' on the surface of the plate. Standard conditions were used in direct ELISA tests, and included using PBS buffer (pH 7.4) and carbonate buffer (pH 9.0), in conjunction with flexiplate ELISA plates. Plates were coated with antigen at concentrations ranging from 1-5 µg/ml, and test, control and Ab samples loaded at a volume of 50 µl. Plates were blocked using BSA blocking buffer (PBS/BSA 1%) for 1 hr, as standard, and washed in-triplicate after each incubation with Ab (primary and secondary) with PBS/Tween20 0.01%. Primary mAbs were generally used at a dilution of 1/2,000-5,000 of the stock concentration (0.8-1 mg/ml, as standard). Secondary Abs were used at a dilution of 1/5,000-10,000.

Secondary Abs were always HRPO-conjugates, so ELISA were developed using a 3,3,5,5,-tetramethylbenzidine (TMB) solution. TMB is a peroxidase sensitive substrate, which when enzymatically cleaved turns blue at a neutral pH. The TMB solution was prepared a minimum of 30 min prior to an ELISA development, by adding 100 µl TMB stock (10 mg/ml TMB diluted in DMSO), and 3 µl H_2O_2 (30% solution) to 9.9 ml phosphate-citrate buffer (51.4 mM Na_2HPO_4, 24.3 mM anhydrous citrate). TMB stock was stored at 4°C between uses. At this temperature, the solution crystallises, so prior to use it must be allowed time to melt at RT. After incubating with the TMB solution for 5-10 min (dependent on the signal from positive and negative controls), reactions were stopped using 10% H_2SO_4 (samples change from blue to yellow, at a low pH). Plates were then read at 450 nm using either 'Labsystems Multiscan Spectrum'

microplate spectrophotometer (Thermo, USA), or ‘SpectraMAX 190’ microplate reader (Molecular Devices, USA).

When screening for CFHR-specific Ab responses in murine tail bleed sera (**Chapter 4**), serum samples were diluted (a 1/2 serial dilution with PBS, starting from 1/5,000) and used instead of a standard primary detection Ab. Therefore, a positive signal would indicate the presence of an Ab positive serum sample. Screening for autoantibodies used the same methodology, but serum was diluted 1/50 with PBS.

Hybridoma screening used the same premise, but instead of sera, tissue culture well SN was applied as the primary detection reagent (diluted 1/2 with PBS). Hybridomas secreting antigen-specific mAbs would be highlighted as positive in the ELISA development.

2.3.4.2 Sandwich ELISA

When attempting to detect soluble proteins in a mixed protein samples (e.g. sera or tissue culture SN), sandwich ELISA was considered the most appropriate method to use. However, sandwich ELISA was only possible when at least two antigen-specific Abs were available. The same conditions were used in this ELISA, as previously described (**Section 2.3.4.1**). However, due to the nature a sandwich ELISA, one of the antigen-specific Abs (the monoclonal) was coated to the plate surface at 5 μg/ml. Secondary detection of the captured antigen was achieved by an antigen-specific pAb. Some secondary Abs were HRPO-conjugated, while others required a tertiary HRPO-conjugate. Secondary (and tertiary) Abs were used at a dilution of 1/5,000-10,000.

2.3.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE gels were used to separate protein samples by molecular weight. Gels were produced in-house using the 'Mighty Small II Gel System' (GE Healthcare, formerly Amersham Biosciences), following the standard method proposed by Laemmli[192]. Stacking and separating gels were made as described in **Table 2.7**. Before the addition of TEMED, solutions were filtered using a 0.22 µM syringe filter.

Stacking Gel	**4%**
1M Tris pH 6.8	0.5ml
30% bis/acrylamide (29:5:1)	0.67ml
dH_2O	2.4ml
10% SDS	40µl
10% APS^	30µl
TEMED*	3µl
Separating Gel	**10%**
1.5M Tris pH 8.8	2.5ml
30% bis/acrylamide (29:5:1)	3.3ml
dH_2O	4.1ml
10% SDS	100µl
10% APS^	50µl
TEMED*	5µl

Table 2.7: SDS-PAGE gel recipe
^Ammonium Persulphate; **N,N,N',N'*-Tetramethylethylenediamine

Protein samples were diluted with '5x Protein Loading Buffer' (0.313M Tris-HCl at pH 6.8, 10% SDS, 0.05% bromophenol blue, 50% glycerol), as non-reduced samples. Reduced samples included 100 mM dithiothreitol (DTT). Reduced and non-reduced samples were boiled at 100°C for 2 min, prior to running. 'PageRuler™ Plus Prestained Protein Ladder' (Fermentas, UK) was loaded as a molecular weight protein marker on each gel.

SDS-PAGE gels were electrophoresed using 'SDS Running Buffer' (25 mM Tris base, 192 mM Glycine, 0.01& SDS, pH 8.3), and running at 30 mA per gel. Gels were stopped when the 'dye-front' reached the bottom of the gel.

2.3.6 Coomassie Staining

Coomassie staining of SDS-PAGE gels was used as a rapid method of visualising all proteins within a given sample. SDS-PAGE gels were incubated with Coomassie blue stain (50% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue) for 30 min, followed by brief washing with dH_2O to remove excess

stain. The stained SDS-PAGE gels were then destained (10% methanol, 10% acetic acid) until the gel was clean and the proteins of interest were visible. Destain incubation time, and destain repeats were specific to each Coomassie stain procedure.

2.3.6 Western Blot

SDS-PAGE separated protein samples were transferred to 'Hybond HCL nitrocellulose' (GE Healthcare) *via* electrophoresis using 'Transfer Buffer' (25 mM Tris base, 192 mM Glycine, 20% Methanol) and running at 100 volts for 1 hr. After transferring, nitrocellulose membranes were blocked for 1 hr with 'Non-fat dried milk (NFDM) Blocking Buffer' (5% NFDM/PBS/0.01% Tween20).

Nitrocellulose membranes were then incubated with primary Ab diluted 1/2000-5000 in 'NFDM Blocking Buffer'. Membranes were then washed for 10 min, in-triplicate, with 'Wash Buffer' (PBS, 0.01% Tween20, pH 7.4). After washing, membranes were incubated with an HRPO-conjugated secondary Ab diluted 1/4,000-10,000 in 'NFDM Blocking Buffer', and incubated for 1 hr. Membranes were then washed as previously described.

The membrane was then immersed in 'Peirce® ECL Western Blotting Substrate' (Thermo Scientific) for 1 min, then wrapped in standard cling-film. The blot was exposure to light-sensitive X-OMAT film in the dark, for increments of time.

When testing for autoantibodies *via* western blot, a 'Strip Blot' methodology was developed. This involved running the antigen of interest in all test wells of the SDS-PAGE gel, and transferring to nitrocellulose as previous described. Once transferred, the nitrocellulose was treated with 'Ponceau Stain' (0.1% Ponceau S w/v, 5% acetic acid) to instantly visualise the protein lanes on the nitrocellulose. The lanes were then cut into strips, placed into in a test tube (with lids) and washed with PBS until the stain had been removed. Strips were then individually blotted with primary and secondary Abs. The 'primary Ab' was patient or control plasma, diluted 1/50 in PBS/0.01% Tween20. After the final wash, strips were lined-up in their original order, with reference to the protein marker. The blot was then developed as previously described.

2.4 Animal Work and Hybridoma Fusions

2.4.1 Murine Immunisations

Female C57BL/6 mice aged 12-16 weeks were selected for immunisation, with the aim of producing an antigen-specific IgG Ab response.

2.4.1.1 Immunisation Protocol and Antigen-Adjuvant Emulsification

Mice were immunised as outlined in **Table 2.8**. The initial CFHR immunisations were administered using Freund's Complete Adjuvant (FCA), followed by subsequent immunisations at week 2 with Freund's Incomplete Antigen (FIA). Antigen was diluted to 0.4 mg/ml in sterile PBS, and a 100 µl volume (40 µg CFHR antigen) was combined with an equal volume of either FCA or FIA. The two solutions were emulsified using two luer-lock glass syringes immediately prior to immunisation.

FCA and FIA emulsions (weeks 0 and 2, respectively) were administered subcutaneously (SC) at either the 'scruff' of the neck, or lower flank. Each antigen-adjuvant immunisation was injected at two sites (100 µl per site). The remaining immunisations were administered intraperitoneally (IP).

Week No.	Immunisation	Buffer/Adjuvant	Injection site	Volume	Antigen load
0	1st	PBS/FCA	SC	200µl	40µg
2	2nd	PBS/FIA	SC	200µl	40µg
4	3rd	PBS/-	IP	200µl	40µg
6	4th	PBS/-	IP	200µl	40µg
8	5th	PBS/-	IP	200µl	40µg

Table 2.8: Murine immunisation protocol

2.4.1.2 Murine Spleen and Blood Collection

Prior to each immunisation, tail bleeds were taken to assess anti-CFHR serum levels. Tail bleeds were taken as previously described[193]. Approximately 50-100 µl of blood was collected per tail bleed. Collected blood was incubated for 1 hr at RT, followed by micro-centrifugation at 13,000 x g for 5 min. Serum was collected, stored on ice and tested on the same day. Sera was then stored at -20°C.

The coagulated blood pellets were discarded. Sera collected prior to the first immunisation served as negative control sera for subsequent tail bleed tests. Murine sera were screened *via* direct ELISA (**Section 2.3.4.1**).

5 d after the final immunisation (week 8), total blood volume was collected *via* cardiac puncture and terminal exsanguination. Blood sera was isolated and stored, as previously described. Spleens were collected and stored on ice in sterile PBS (pH 7.4), and processed within 1 hr of collection.

2.4.2 Hybridoma Fusion

Spleens were transferred to 1 well of a 6-well tissue culture plate (surface area 9.6cm^2), with 5 ml sterile PBS. Spleens were crushed using two frosted glass slides, releasing the splenocytes into solution. Splenocytes were resuspended by gentle aspiration with a pipette, and transferred to a 50 ml tube, followed by the addition of 20 ml ice cold unsupplemented RPMI 1640 media (PAA, UK). The cell suspension was stored on ice for 5 min, to allow large debris to settle to the bottom of the tube. The cell suspension was carefully aspirated (leaving unwanted debris behind) and transferred to a new 50 ml tube. At this stage 10^7 NS0 cells were collected from standard incubation treated following the same protocol as the splenocytes.

Splenocytes and NS0 cells were centrifuged at 500 x g for 5 min at 4°C. After centrifugation, the SN were discarded and pellets resuspended into a further 20 ml of ice cold unsupplemented media. The cell suspension was centrifuged as before. This wash procedure was repeated in-triplicate. The final cell pellets were resuspended in 20 ml pre-warmed (37°C) unsupplemented RPMI media. The cell suspensions were then combined and centrifuged at 500 x g for 5 min at RT. The SN was discarded. The mixed cell pellet was gently resuspended by repeatedly tapping the bottom of the tube. At this stage, the cells were ready for the fusion procedure.

A sterile 50% PEG 1500 solution was pre-warmed to 37°C, and 1.5 ml loaded into a 2 ml syringe, with needle. The warm 50% PEG 1500 solution was then added ‘drop-wise’ over 2 min to the NS0/splenocyte mixture, with continuous gentle tapping of the tube. After 2 min, the cells were immediately resuspended with pre-warmed 20 ml supplemented RPMI media, centrifuged at 500 x g for 5

min at RT, the SN discarded and the pellet resuspended again. This was repeated in-triplicate, to remove excess PEG 1500. The final pellet was resuspended in 195 ml supplemented RPMI media, with the addition of HAT supplement for hybridoma selection (**Section 2.2.1**). The hybridoma fusion cell suspension was transferred to 10 x 96 well tissue culture plates (200 µl per well). The remaining 'excess' culture was split across the final plate to ensure all potential hybridomas were included. The remaining subcloning protocol is summarised later in this chapter (**Figure 2.3)**, and the ELISA specificity screening was completed as previously described (**Section 2.3.4.1**).

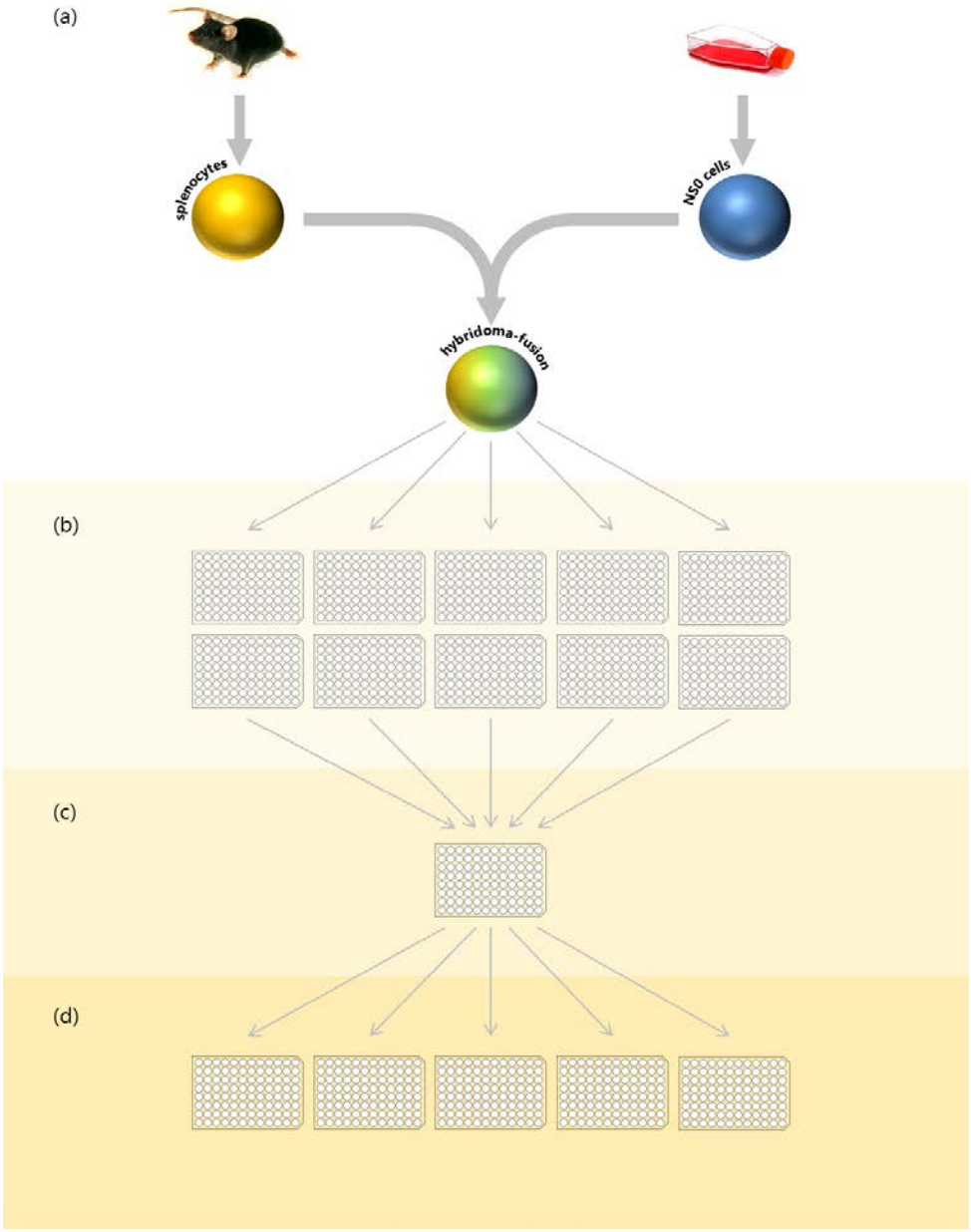


Figure 2.3: Hybridoma screening protocol

(a) An immunised C57BL/6 mouse spleen is collected and splenocytes are released into suspension. Splenocytes and NS0 cells are then counted and prepared for PEG-fusion. Splenocytes and NS0 fusion-partner cells are fused using 50% PEG-1500. **(b)** Following the fusion procedure, the hybridoma fusion culture is plated out across 10 x 96-well tissue culture plates. The hybridoma cultures are incubated for 7-10 d. **(c)** All hybridoma wells are visually screened and wells containing clone colonies are selected and condensed into individual wells of a 96-well plate (using fresh media). The selected colonies plate is incubated for 3 d, the screened via direct ELISA to identify rCFHR-specific Ab secretion. **(d)** Positive hybridoma wells are selected and subjected to a limiting dilution across a 96-well plate. The selected positive hybridoma limiting dilution cultures are incubated for 7-10 d, and re-screened as before. This procedure is repeated until 3 limiting dilutions have been completed for each original positive well selected from stage **(c)**.

2.5 Autoantibody Screening

2.5.1 Patient and Control Plasma

To assess the presence of CFH family autoantibodies in the normal population, human plasma was collected from 100 ‘normal’ blood donor controls. Blood samples were supplied by the Blood Transfusion Service (Newcastle-upon-Tyne, UK), and plasma and genomic DNA collected courtesy of the NHS sample reception at the Centre for Life (Newcastle-upon-Tyne, UK). These samples will be referred collectively as ‘BTS’ control plasma.

The BTS controls are made up of a 1:1 ratio of male to female donors. The ethnicity of donors was 98% white and 3% black Afrocarribean. Donor age ranged from 17-72 years. The aHUS plasma samples came from the Newcastle patient cohort [139,162], and IgAN samples were collected as part of a new cohort IgAN patients. Ethical approval for the use of these samples was given by the NRES Committee North East – Northern and Yorkshire (Genetic factors predisposing to the haemolytic uraemic syndrome, MREC 01/3/083) and NRES Committee North East – Newcastle and North Tyneside 1 (Complement and glomerulonephritis – genetic factors, 05/Q0905/158).

2.5.2 Preliminary ELISA Testing

The generation of a robust and sensitive ELISA screen for autoantibodies in patient samples requires comprehensive optimisation. Therefore, preliminary testing of ELISA parameters was undertaken, including ELISA plate surface, antigen coating buffer, antigen coat concentration and plate surface blocking. These parameters were evaluated in a multiplex testing process (**Figure 2.4**).This enabled rapid production of a stable ELISA screen for each CFHR antigen used, and for each autoantibody isotype being tested (IgG and IgA). Multiplex testing was done in 2 stages, as will now be described.

Stage 1 is designed to determine the most appropriate conditions for optimal coating of the antigen, assessing coat surface x coat buffer x coat antigen concentration. A primary Ab was used to assess the efficiency of antigen binding, where the highest signal is an indication of the best conditions for the

specific coating antigen. The blocking reagent used in ‘Stage 1’ is kept consistent across all conditions.

Stage 2 is designed to determine the most appropriate conditions when BTS control plasma (assumed negative) is included as in the position of ‘primary Ab’, as autoantibodies will essentially serve as ‘primary’ in the final ELISA screen. The aim of ‘Stage 2’ is to choose conditions where the mean BTS control value (from 10 samples) approaches zero, while maintaining a high positive control value. The positive control is an autoantibody positive plasma sample, previously determined (if available). This control is available for rCFHR1 screening, due to the identification of positivity from CFH autoantibody testing (IgG isotype), and fragment analysis highlighting C-terminal binding of CFH and CFHR1[162]. However, when screening rCFHR2-5, or for IgA autoantibodies to all CFH family proteins, no known positive control was available.

‘Stage 2’ uses the best conditions for antigen coating, as determined by ‘Stage 1’ results. These conditions primarily include coat buffer, and coat antigen concentration. Plate surface is reassessed in ‘Stage 2’ as different blocking reagents are tested. Therefore, it is important to reassess the influence of plate surface on each of the blocking reagents. Therefore, ‘Stage 2’ assesses coat surface x blocking reagent x donor plasma.

(a)

		Conc 1 0.1µg/ml	Conc 2 0.5µg/ml	Conc 3 1µg/ml	Conc 4 2.5µg/ml	Conc 5 5µg/ml	Conc 6 10µg/ml
Plate 1 Flexiplate	Coat Buffer 1 PBS						
	Coat buffer 2 'Coating Buffer'						
	Coat buffer 3 Carbonate						

		Conc 1 0.1µg/ml	Conc 2 0.5µg/ml	Conc 3 1µg/ml	Conc 4 2.5µg/ml	Conc 5 5µg/ml	Conc 6 10µg/ml
Plate 2 Perkins Elmer	Coat Buffer 1 PBS						
	Coat buffer 2 'Coating Buffer'						
	Coat buffer 3 Carbonate						

		Conc 1 0.1µg/ml	Conc 2 0.5µg/ml	Conc 3 1µg/ml	Conc 4 2.5µg/ml	Conc 5 5µg/ml	Conc 6 10µg/ml
Plate 3 Maxisorb	Coat Buffer 1 PBS						
	Coat buffer 2 'Coating Buffer'						
	Coat buffer 3 Carbonate						

(b)

		BTS1	BTS2	BTS3	BTS4	BTS5	BTS6	BTS7	BTS8	BTS9	BTS10
Plate 1 Flexiplate	Blocking Buffer 1 PBS/BSA 1%										
	Blocking Buffer 2 1/5 Ultrablock										
	Blocking Buffer 3 PBS/Tween20 0.1%										

		BTS1	BTS2	BTS3	BTS4	BTS5	BTS6	BTS7	BTS8	BTS9	BTS10
Plate 2 Perkins Elmer	Blocking Buffer 1 PBS/BSA 1%										
	Blocking Buffer 2 1/5 Ultrablock										
	Blocking Buffer 3 PBS/Tween20 0.1%										

		BTS1	BTS2	BTS3	BTS4	BTS5	BTS6	BTS7	BTS8	BTS9	BTS10
Plate 3 Maxisorb	Blocking Buffer 1 PBS/BSA 1%										
	Blocking Buffer 2 1/5 Ultrablock										
	Blocking Buffer 3 PBS/Tween20 0.1%										

Figure 2.4: Testing parameters for the autoantibody screening ELISA

The various parameters of each autoantibody ELISA were tested to select the most suitable parameters for each screen. This optimisation process was split into two stages. **(a)** Stage 1 involved testing ELISA plate surface (Flexiplate, Perkins Elmer and Maxisorb), antigen coating buffer (PBS pH 7.4), 'Coating Buffer' pH 7.4, and Carbonate buffer pH 9.0) and a range of CFHR coating concentrations (0.1-10 µg/ml). **(b)** Stage 2 involved testing plate surface (as before), blocking buffer (PBS/BSA 1%, 'Ultrablock' diluted 1/5, and PBS/Tween20 0.1%) and a cohort of 10 BTS normal controls (plasma diluted 1/50). All BTS plasma was duplicate-tested on no coat control plates to assess background level binding to the blocking buffer. The background binding was then subtracted from the coated plate result.

2.6 Statistical Analysis

2.6.1 General Analysis

When conducting ELISA, samples were loaded in-duplicate/triplicate and the mean value established for presentation of results. Under these circumstances, the standard deviation was applied to measure the spread around the mean (presented as positive error bars). The standard deviation values were calculated using the follow equation.

$$\sigma = \sqrt{\frac{\sum(\bar{x} - x)^2}{n - 1}}$$

2.6.2 Analysis of autoantibody data

The disease and control data from autoantibody screening was not analysed statistically to assess the significance of a change between the two groups. The design of the experiment was optimised towards creating a base-line negative result, whereby the distribution of data due to positive results would skew the data to the right of a Gaussian distribution. If the data were to be analysed, a Fisher's exact test would be considered due to the quadranted presentation of results (i.e. patient and control groups x positive or negative results).

2.6.3 Analysis of CFHR4 plasma levels

CFHR4 concentration levels were assessed in small preliminary populations of aHUS and BTS controls. Each population consisted of 10 samples, so a Gaussian distribution could not be determined. Therefore, a nonparametric Mann-Whitney test was selected to analyse the statistical significance of a change between the two populations. Data was analysed using GraphPad Prism® version 5.0 (GraphPad Software Inc.)

Chapter 3

3. Production & Purification of Recombinant CFHR Proteins

3.1 Introduction

CFH and the CFHR proteins are a group of highly homologous proteins, of which CFH and CFHR1 are considered to exhibit potent complement regulatory activity[153,194-196]. CFHR4 has recently been shown to play a supportive role in the activation of C3 convertase[168]. The functional role of CFHR2, CFHR3 and CFHR5 remain to be elucidated. However, there is sufficient evidence to suggest potential roles for CFHR3, CFHR4 and CFHR5 in the pathogenesis of renal diseases such as aHUS[162] and CFHR5 nephropathy[197].

The role of the CFHR proteins remains poorly understood, in contrast to our knowledge of CFH in health and disease[198-200]. At the beginning of this research project, there were no commercially available CFHR proteins, or mAbs for specific detection or isolation of CFHR proteins from human serum. Therefore, in order to study CFHR proteins, a full panel of recombinant proteins was deemed essential, with the subsequent aim to use these to generate new mAbs and pAbs. These combined reagents would allow a complete and thorough characterisation of the role of CFHRs in health and disease.

3.2 Chapter 3 Aims

To produce five DNA plasmid constructs containing cDNA for each of the five *rCFHR* genes, followed by production in mammalian cell culture.

To generate highly pure rCFHR proteins to enable future studies, including the development of mAbs.

3.3 Results

3.3.1 Modification of *pDEF(nMCS)* Expression Vector

The DNA expression vector *pDEF(nMCS)* has been commonly used in conjunction with CHO cells to produce a range of human proteins, including human complement proteins[185-188]. The vector contains an *elongation factor 1α* (*eF1α*) promoter region upstream of the multiple cloning site (MCS) which results in constitutive protein expression when transfected into a mammalian cell system. The vector also contains the hygromycin B resistance gene [189,190], which allows selection of transfected cells using hygromycin B supplemented media. Thus, *pDEF(nMCS)* was chosen as our base construct.

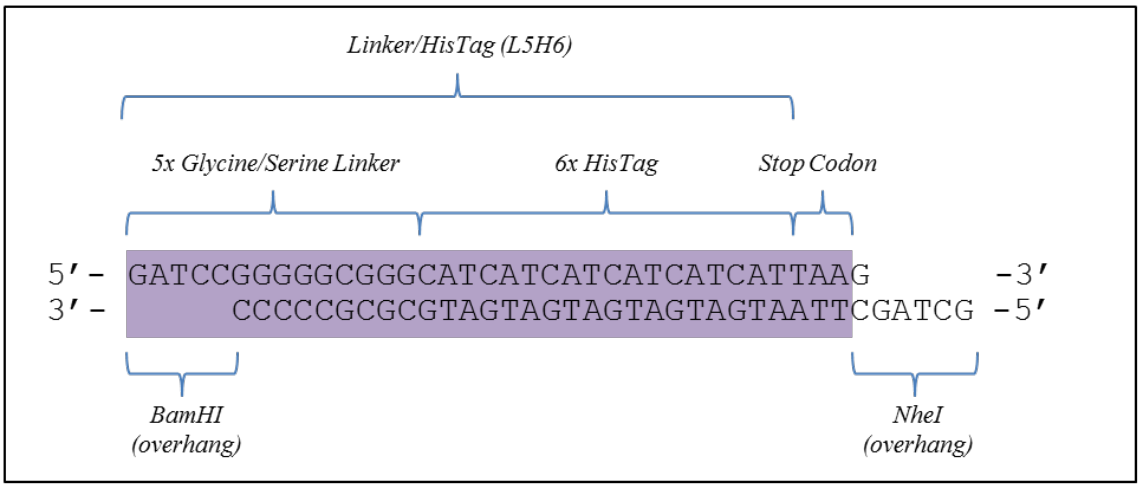
Before the insertion of the *rCFHR* cDNA insert, *pDEF(nMCS)* was modified to include a sequence coding for a 5 amino acid linker, followed by a 6 histidine purification tag (HisTag). The linker-HisTag (*L5H6*) sequence was incorporated immediately downstream of the restriction enzyme (RE) site used for ligation of each *rCFHR* insert.

The aim of the modification was to facilitate the addition of *L5H6* to the 3' end of each inserted *rCFHR* construct. Two complementary single stranded DNA (ssDNA) oligomers were designed encoding for the *L5H6* sequence, with the addition of *BamHI* and *NheI* RE sites at either end. The complementary oligomers were designed to anneal leading to *BamHI* and *NheI* overhangs, as would be achieved after a RE digestion of *pDEF(nMCS)* (**Figure 3.1a**).

After insertion of the annealed *L5H6* fragment into *pDEF(nMCS)*, OneShot® Top10 *E.coli* (Invitrogen) were transformed with 5 µl of the ligation reaction and propagated as previously described (**Section 2.1.6**).

Positive colonies were selected on ampicillin 200 µg/ml supplemented LB agar plates, picked and cultured overnight. Plasmid DNA was isolated *via* standard column DNA preparation according to manufacturer's instruction (QIAprep Miniprep Kit, Qiagen). Correct insertion of *L5H6* was confirmed *via* DNA sequencing of the *pDEF(nMCS)* (**Section 2.1.2**). The DNA sequencing results show successful insertion of the *L5H6* fragment into one of the positively selected bacterial clones (**Figure 3.1b**). The modified vector will be referred to as *pDEF(L5H6).*

(a)



(b)

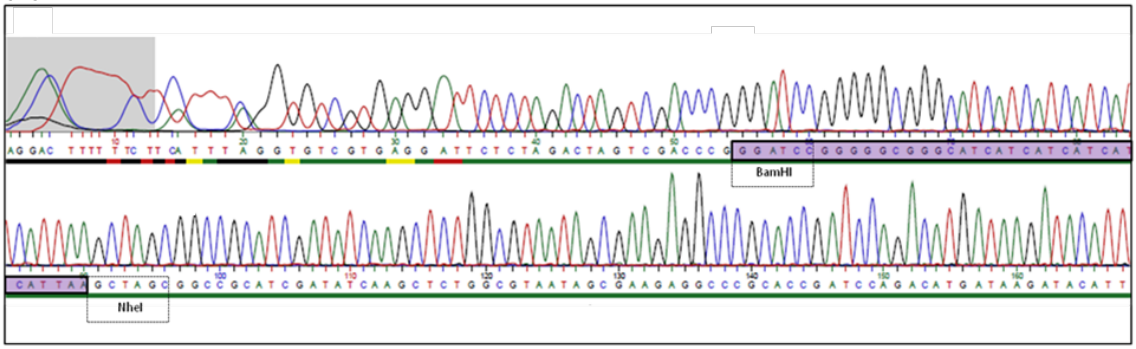


Figure 3.1: Construction of *pDEF(L5H6)*

(a) Illustration of the annealed complementary single stranded DNA oligonucleotides that were designed to produce a double stranded DNA fragment (*L5H6*) with *BamHI* and *NheI* overhangs. **(b)** DNA sequencing data of the newly generated *pDEF(L5H6)* vector showing the successful insertion of the *L5H6* fragment (purple shaded area). The *BamHI* and *NheI* sites are listed and highlighted by boxes.

3.3.2 PCR Amplification of *rCFHR* cDNA

Plasmids containing cDNA for each of the *CFHR* genes were obtained from different sources (**Table 2.2**). Upon receipt of each clone, DNA sequencing was completed to clarify the correct DNA sequence had been supplied. These sequencing results are discussed further in **Section 3.3.4**.

Template DNA for each *rCFHR* construct was amplified using specifically designed primers (**Table 2.1**). Amplified cDNA was visualised on an ethidium bromide supplemented 1% agarose/TAE gel (**Figure 3.2**). The results show successful PCR amplification of *rCFHR1* (990bp), *rCFHR2* (810bp), *rCFHR3* (990bp), *rCFHR4B* (991bp), and *rCFHR5* (1707bp). The PCR primers were designed specifically for cloning of *CFHR* cDNA so no specific positive control for primer function (under these specific PCR conditions) could be included with these PCR reactions. However, negative controls (excluding primers and/or DNA template) were included in all PCR reactions and remained negative as expected (control results not shown). PCR cloned *rCFHR* cDNA was purified from the PCR mixture (QIAquick PCR Purification Kit, Qiagen) and DNA concentration was determined *via* UV spectrophotometry.

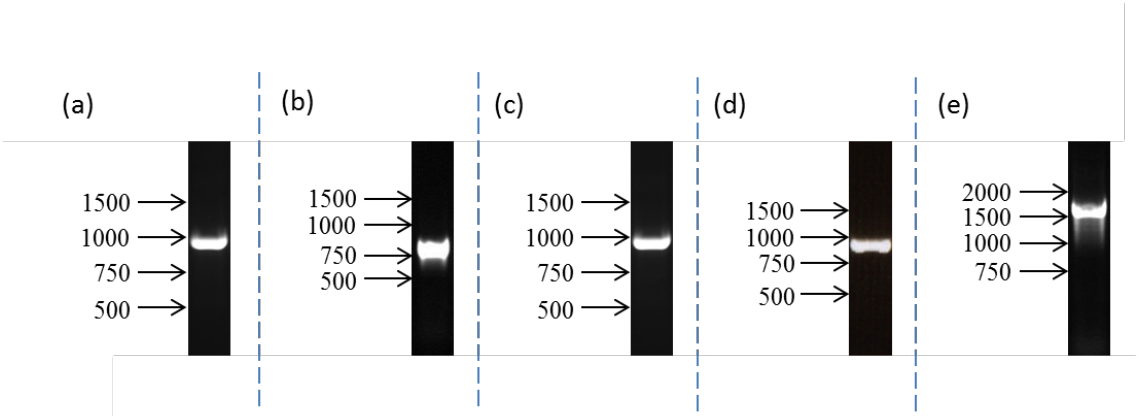


Figure 3.2: *rCFHR* cDNA PCR

Samples from various PCR cloning reactions were ran on 1% TAE/agarose gels, supplemented with ethidium bromide. Shown are examples of the PCR reactions for **(a)** *rCFHR1* (expected size 990bp), **(b)** *rCFHR2* (expected size 810bp), **(c)** *rCFHR3* (expected size 990bp), **(d)** *rCFHR4B* (expected size 993bp), and **(e)** *rCFHR5* (expected size 1707bp). These results are representative of many such reactions. Negative controls lanes remained completely blank and have been omitted.

3.3.3 Construction of *pDEF/rCFHR*

Purified *rCFHR* DNA was double digested and ligated into linearised *pDEF(L5H6)*, as before. Again, 5 µl of ligation reaction was transformed into One Shot® Top10 *E.coli*. Single colonies were selected and whole cell DNA was collected as previously described (**Section 2.1.3.2**) and used as template DNA in a colony PCR procedure. The colony PCR results for *pDEF/rCFHR1*, *rCFHR3* and *rCFHR4B* show amplification of a product of the expected size for each *rCFHR* cDNA insert (**Figure 3.3**). Negative results show a band of 192bp, the distance between the *pDEF(nMCS)* forward and reverse primers.

Bacterial colonies identified as positive *via* colony PCR were cultured overnight in 5 ml LB broth, supplemented with 200 µg/ml ampicillin. Plasmid DNA was isolated and purified, as previously described, and each *pDEF/rCFHR* plasmid was double digested using *XbaI* and *BamHI* (*rCFHR1-4*), or *SpeI* and *BamHI* (*rCFHR5*). Restriction endonuclease digested plasmids were electrophoresed as previously described. Linearised DNA bands of the expected sizes; *pDEF(L5H6)* vector (~7kb), and the linearised *rCFHR* inserts: *rCFHR1* (990bp), *CFHR2* (810bp), *CFHR3* (990bp), *CFHR4B* (991bp), and *CFHR5* (1707bp) are clearly visible in the respective panels (**Figure 3.4**). Each panel contains a restriction endonuclease digestion of *pDEF(L5H6)*, loaded as a negative control. This negative results show a linearised vector band of ~7kb, without a MCS *rCFHR* ‘drop-out’ product.

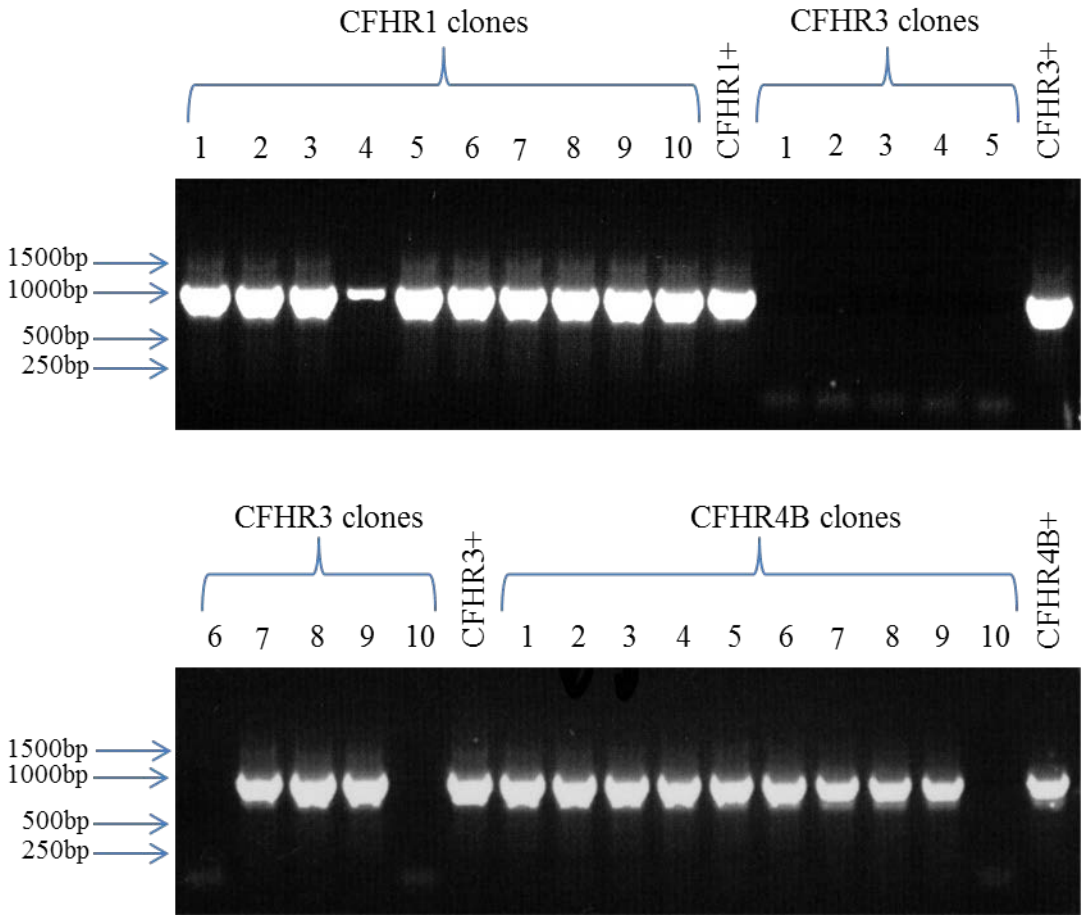


Figure 3.3: Colony PCR

Single bacterial colonies (10 clones per *pDEF/rCFHR* transformation) were selected and total cell DNA collected and used as template in colony PCR. Primers were designed to amplify the contents of the MCS. PCR reactions were electrophoresed on 1% agarose gels, supplemented with ethidium bromide. Colony PCR results are shown for transformations for *pDEF/rCFHR1, rCFHR3* and *rCFHR4B* as indicated above the lanes. Each lane represents a colony PCR result from a single bacterial colony. Positive controls for each construct (CFHR1+, CFHR3+ & CFHR4B+), using purified *pDEF/rCFHR* plasmid DNA as template. These data are a representative of the *pDEF/rCFHR* colony PCR runs.

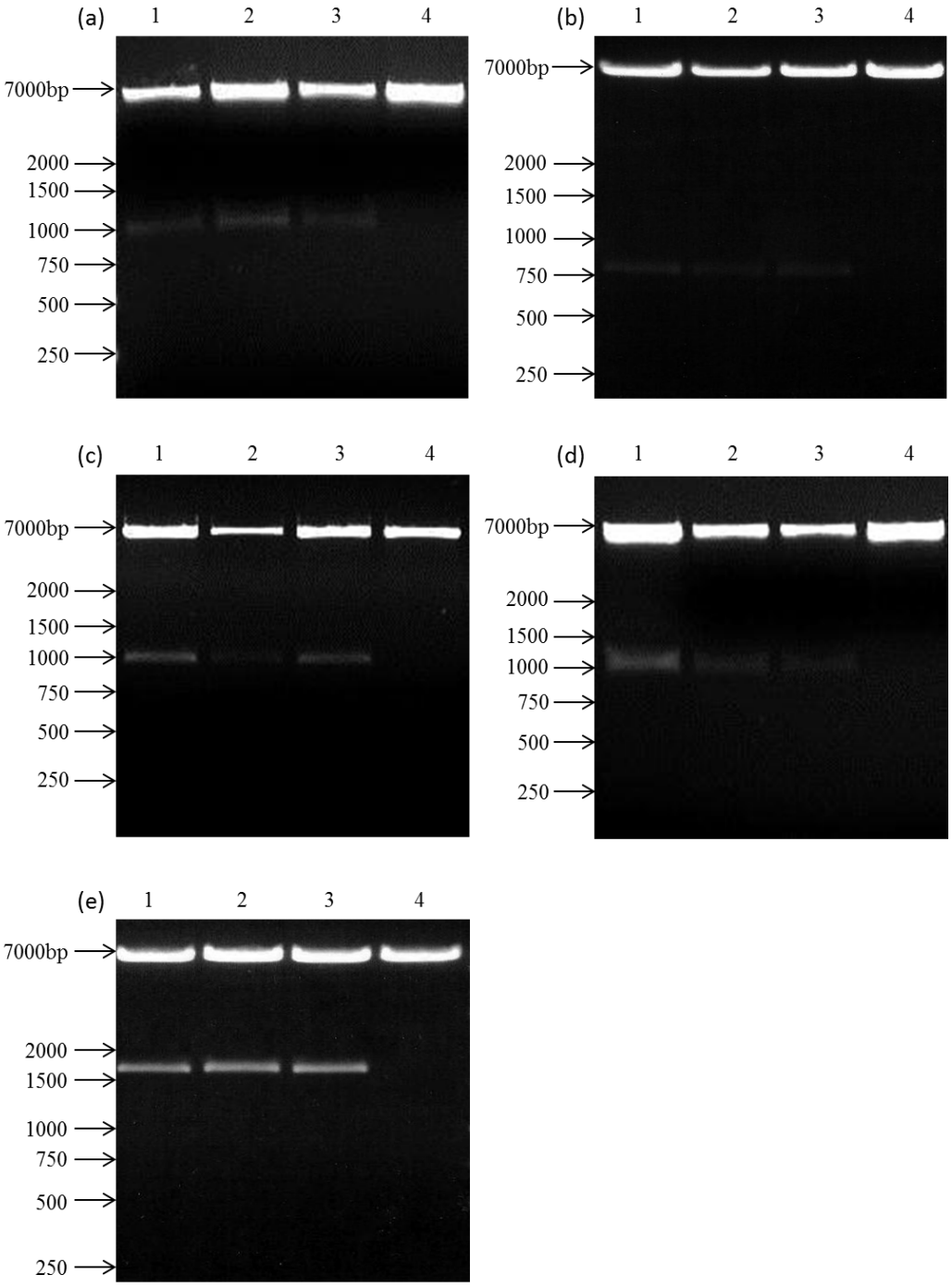


Figure 3.4: *pDEF/rCFHR* DNA construct digestions

Plasmid DNA was isolated from small batch cultures of ampicillin resistant bacterial tranformant colonies, and restriction enzyme digested with *XbaI* and *BamHI* (*pDEF/rCFHR1-4*), or *SpeI* and *BamHI* (*pDEF/rCFHR5*). In panels a-e, lanes 1-3 is plasmid DNA isolated from randomly selected positive colonies, and lane 4 is double digested *pDEF(L5H6),* as a negative control. **(a)** *pDEF/rCFHR1*; **(b)** *pDEF/rCFHR2*, **(c)** *pDEF/rCFHR3*, **(d)** *pDEF/rCFHR4B*, and **(e)** *pDEF/rCFHR5*.

3.3.4 DNA Sequencing of *pDEF/rCFHR* constructs

PCR *rCFHR* templates were initially sent for DNA sequencing (**Section 3.3.2**) to validate the correct cDNA insert had been supplied. Results from forward and reverse sequencing of template DNA were aligned alongside *rCFHR* reference sequences obtained from the NCBI nucleotide database (www.ncbi.nlm.nih.gov/nucleotide). The *CFHR* cDNA reference accession numbers are as follows: *rCFHR1*; NM_002113, *rCFHR2*; NM_005666, *rCFHR3*; NM_021023.4, *rCFHR4B*; NM_006684, and *rCFHR5*; NM_030787. Sequence alignments were completed using Vector NTI v.10 (Invitrogen). Sequence alignments for *rCFHR1*, *rCFHR2*, and *rCFHR3* (Appendix I) share 100% homology between the reference sequence and template sequencing results. However, the alignments for *rCFHR4B* and *rCFHR5* highlighted nucleotide sequence variants (**Figure 3.5**). Sequencing of *rCFHR4B* showed 6 nucleotide sequence variants (*156T>C, 171T>C, 216T>C, 231T>G, 243G>A,* 237A>G). These variants do not result in translational amino acid substitutions, so from a functional perspective, these are 'silent' nucleotide variants. However, the *rCFHR4B* sequence also contained a sequence variant (236C>T) which translated to a 79Ser>Leu amino acid change. All sequence variants were assessed using Alumut v.6 database, courtesy of the Institute of Genetic Medicine, Newcastle University.

From a genetic perspective, it is interesting that all *rCFHR4B* nucleotide changes cluster to a 10% region of the *rCFHR4B* cDNA sequence (mapping to SCR1). Whether there is anything significant to note from this observation is currently unclear.

Sequencing of *rCFHR5* showed two nucleotide sequence variants (609A>G, 1122A>G) which did not result in any amino acid substitutions. The 1122A>G variant is located 2bp from the 5' splice site of exon 7, which may have an effect on RNA splicing of the *CFHR5* transcript. However, this should not cause difficulty for this construct as transcript splicing is already complete (working with *rCFHR5* cDNA).

To validate the correct insertion of each *rCFHR* clone into *pDEF/L5H6*, each of the *pDEF/rCFHR* constructs were also DNA sequenced using the same primers

as used previously for colony PCR. The *pDEF/rCFHR* sequencing results were aligned with the reference and template sequences. The results were identical to the template sequence, indicating that no sequence changes had been introduced during the bacterial cloning process.

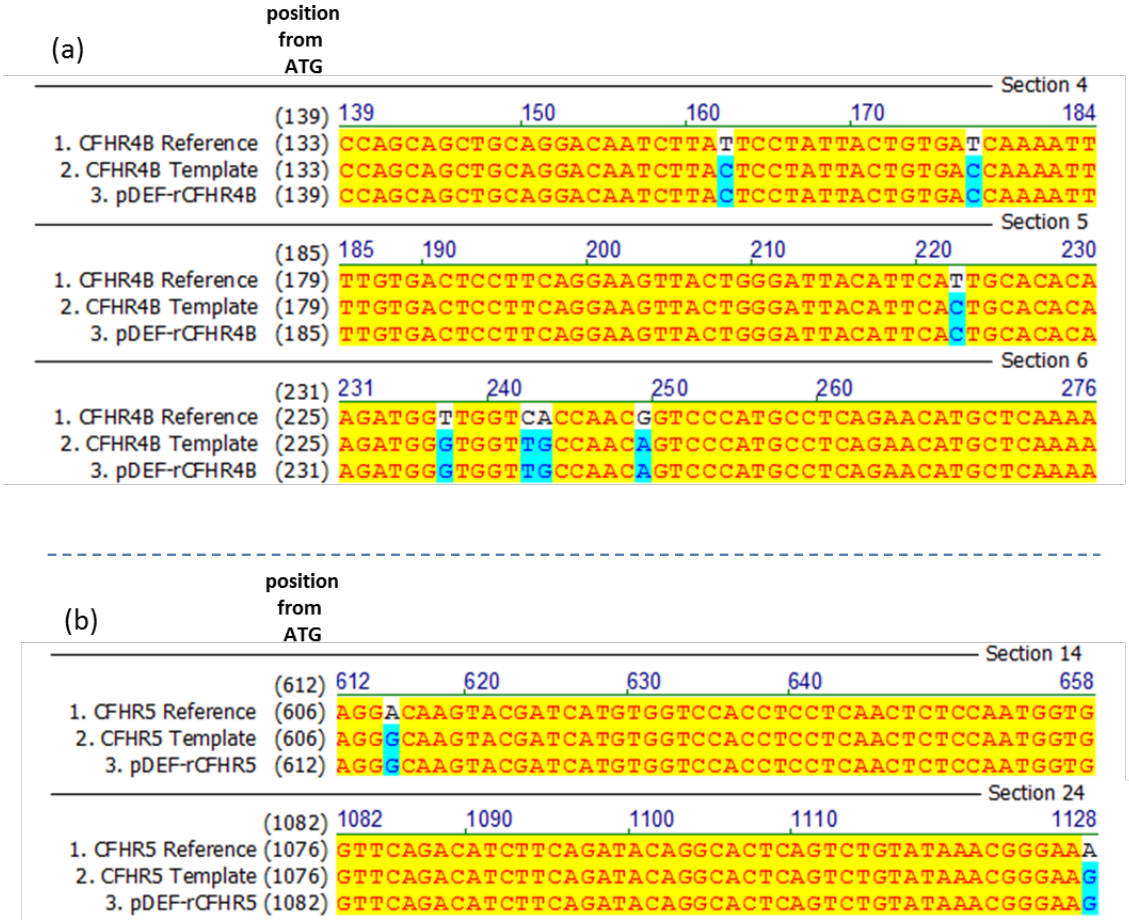


Figure 3.5: DNA sequencing of *rCFHR* constructs

rCFHR cDNA was DNA sequenced prior to PCR amplification (sequencing of PCR template), and again after bacterial transformation (final *pDEF/rCFHR* construct). A cDNA reference sequence was used for each *rCFHR (NCBI database)*. The reference, template and *pDEF/RCFHR* sequences were aligned and nucleotide variants analysed. Sequence homology and nucleotide variant are highlighted (yellow and cyan, respectively). **(a)** DNA sequence alignment using Vector NTI software for regions of *rCFHR4B* where nucleotide variants were found, when comparing the reference and template sequences. **(b)** DNA sequence alignment for the regions of *rCFHR5* where nucleotides variants were found when comparing the reference and template sequences.

3.3.5 Protein expression and CHO transfectant sub-cloning

After transfecting the *pDEF/rCFHR* constructs into wildtype CHO cells using jetPEI® transfection reagent (**Section 2.2.2.1**), tissue culture growth media was collected after 10 d and tested for the presence of each rCFHR protein. For each rCFHR transfection, 12 individual clone colonies were selected, and growth media from each clone was tested *via* sandwich ELISA. An anti-HisTag Ab was coated to ELISA plates, followed by the addition of cell culture SN. HisTag-bound rCFHR proteins were detected using a goat anti-CFH pAb, with subsequent detection using a HRPO-conjugated donkey anti-goat IgG, as previously described (**Section 2.3.4.2**).

A positive result was considered as a UV absorbance reading of more than 3 times the signal of the negative control cell culture SN (wild type CHO cell growth media). The results indicate 9 of the 12 clones analysed in the rCFHR1 transfected clones yielded a positive result (**Figure 3.6a**). Clone R1.4 yielded the highest absorbance reading, suggesting this clone as producing the highest levels of rCFHR1. Therefore, R1.4 was selected for further analysis and carried forward for large batch culturing.

Using the same approach, clones R3.2, R4.2 and R5.5 were selected and carried forward for large batch culturing (**Figure 3.6b-d**).

The expression of rCFHR2 could not be detected in tissue culture growth media, either *via* ELISA or western blot (**Figure 3.7**). This was compounded by the lack of a suitable positive control. Human serum is a source of native CFHR2, but detection using the anti-CFH pAb had previously been unsuccessful in western blot analysis (data not shown). Detection of rCFHR2 was attempted *via* sandwich ELISA using an anti-HisTag mAb coated plate (as previously described to capture rCFHR3, rCFHR4B and rCFHR5), but no positive signal could be detected in any of the R2.1-12 monoclonal cell clones (**Figure 3.7a**). However, the positive control protein (a recombinant CFH fragment, consisting of SCRs 16-18, with 6*HisTag at the N-terminus) was clearly detectable in the sandwich ELISA. It is unclear whether this is due to a deficiency in rCFHR2 protein expression, or a result of poor binding affinity of the anti-CFH polyclonal. The anti-HisTag monoclonal was also used as a

primary Ab in a western blot analysis, but the anti-HisTag mAb was unsuccessful at detecting rCFHR2 in tissue culture SN (**Figure 3.7b**). The anti-HisTag Ab was functional in the western blot, as a HisTag positive control signal was present. However, this primary Ab was unable to detect any of the histidine-tagged rCFHR proteins produced.

As a result of the issues with detection (or expression) of rCFHR2, work on rCFHR2 was placed on hold until a suitable detection reagent became available.

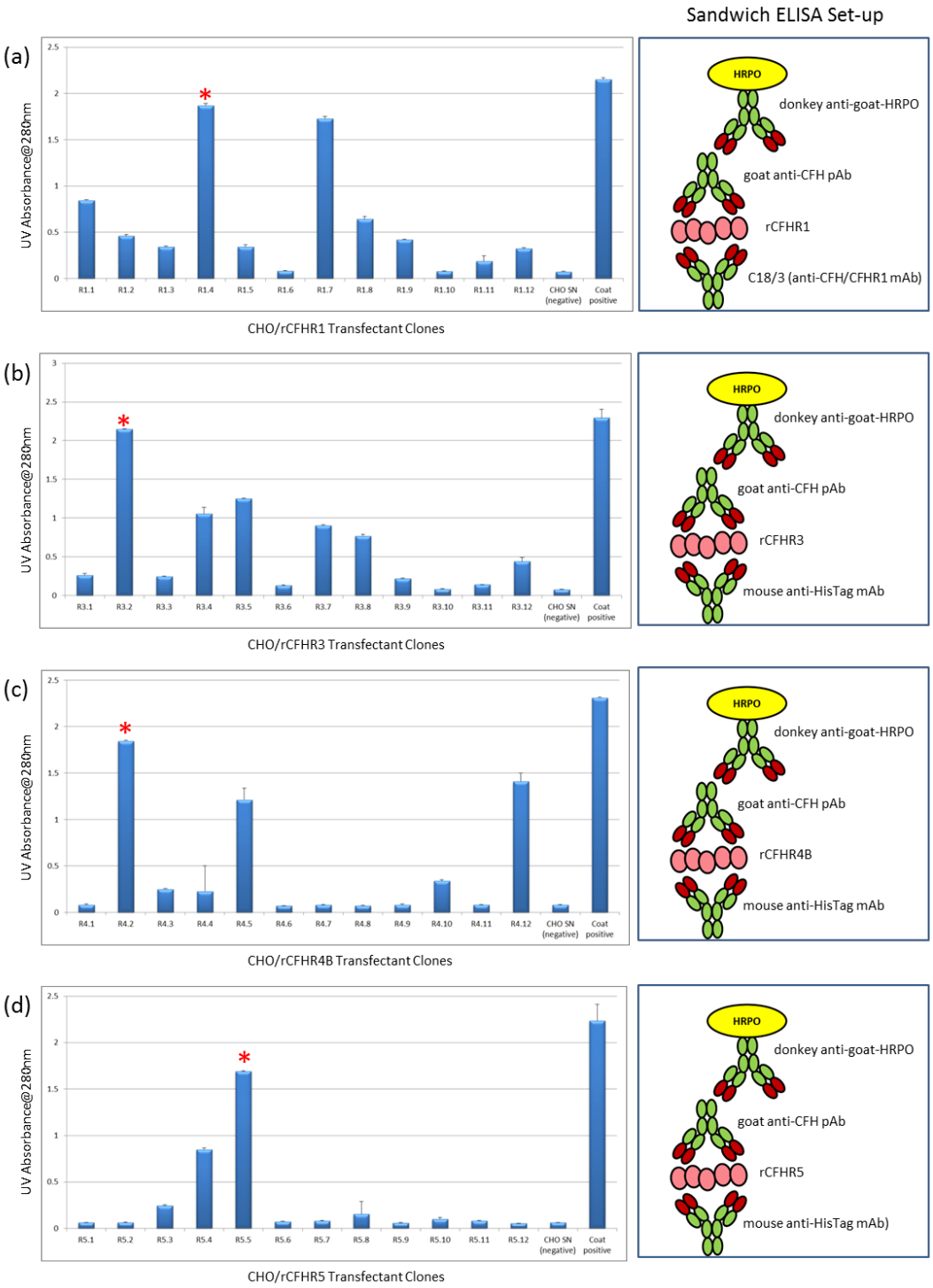


Figure 3.6: CHO/rCFHR transfectant colony testing

12 clones were selected for each of the four CHO/rCFHR (1, 3, 4B and 5) and d +7 growth media applied to ELISA plates coated with a capture Ab. For rCFHR1 detection, the plate was coated with C18/3 mAb (100 µl at 5 µg/ml), and plates for rCFHR3, rCFHR4B or rCFHR5 were coated with anti-HisTag mAb (100 µl at 5 µg/ml). All plates were then probed with an anti-CFH pAb (1/5,000 dilution), followed by probing with a HRPO-conjugated anti-goat IgG. A positive signal was determined as being 3 times the signal of the CHO SN negative control. Clones with the highest signal, which were selected and carried forward for large batch production of each rCFHR protein, are marked with an asterix (*).

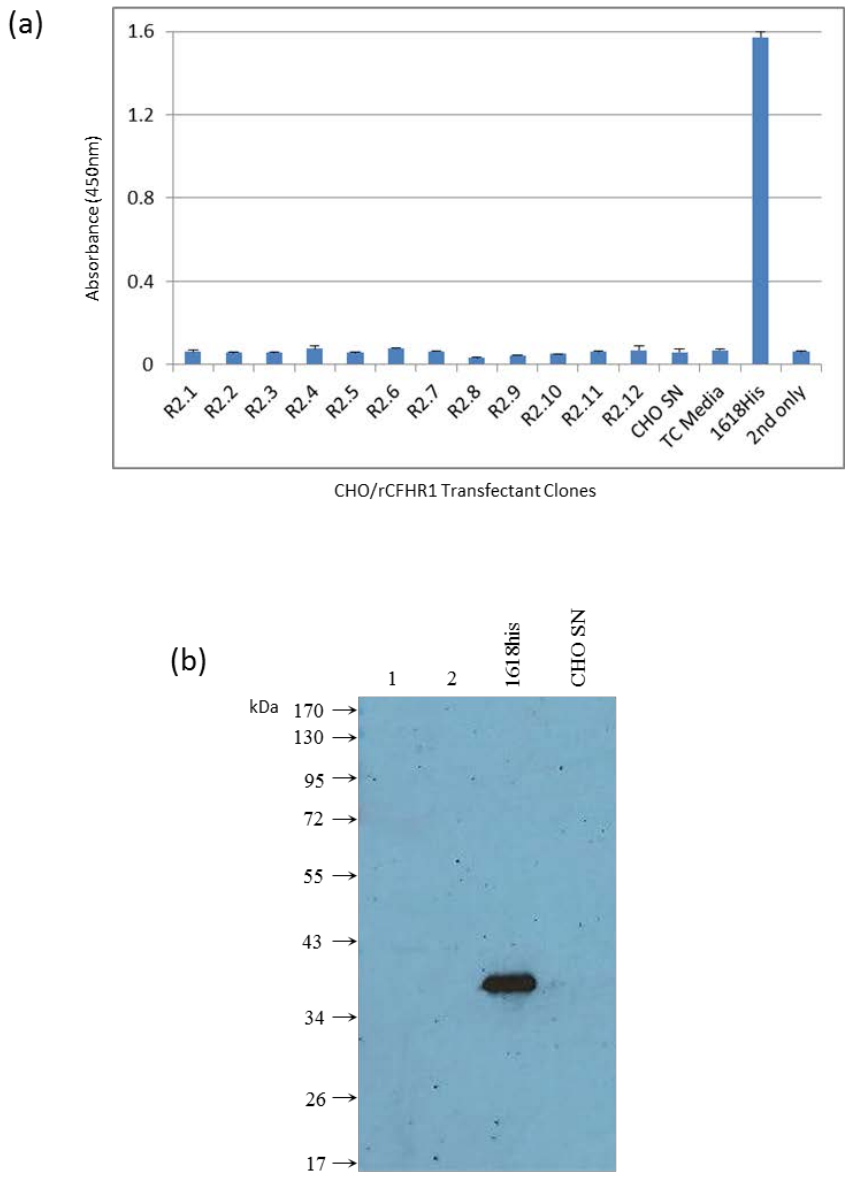


Figure 3.7: rCFHR2 protein detection in CHO/rCFHR2 transfectants

(a) 12 CHO/rCFHR2 clones were selected and d +7 SN applied to ELISA plates coated with an anti-HisTag mAb (100 µl at 5 µg/ml). The ELISA was then probed with an anti-CFH pAb (1/5,000 dilution), followed by probing with a HRPO-conjugated donkey anti-goat IgG. A positive signal was determined as being 3 times the signal of the CHO SN negative control. **(b)** CHO/rCFHR2 transfectant SNs were separated using Tris-Glycine SDS-PAGE (10% gel) and transferred to nitrocellulose membrane. Membranes were incubated with an anti-HisTag mAb, followed by detection using an HRPO-conjugated sheep anti-mouse IgG secondary pAb. Lane 1: 10 µl rCFHR2 tissue culture SN; lane 2: 20 µl rCFHR2 tissue culture SN; lane 3: HisTag positive control protein (1618his; CFH fragment consisting of SCR domains 16-18); lane 4: CHO cell tissue culture SN (CHO SN) as a negative control.

3.3.6 Purification of rCFHR Proteins

As previously discussed, all rCFHR proteins were designed with a C-terminal HisTag for rapid purification *via* a nickel coated chelate chromatography column. Tissue culture SN from each clone (CHO/rCFHR1.4, CHO/rCFHR3.2, CHO/rCFHR4.2 and CHO/rCFHR5.5) was collected (1-2 litres) and diluted in '2x His Binding Buffer' (**Section 2.3.1**). Pilot purification established that 20 mM imidazole in the binding buffer was sufficient to restrict non-specific protein binding to the nickel chelating column, with the exception of rCFHR1 purification; this protein preparation favoured a 40 mM imidazole in the binding buffer. rCFHR proteins were then eluted using an imidazole gradient from 20 or 40 mM (His Binding Buffer) to 500 mM imidazole (His Elution Buffer). 1 ml fractions were collected throughout the elution, and examples of the UV absorbance readings for each rCFHR protein are presented (**Figure 3.8**).

A double absorbance peak is visible during the imidazole gradient elution of rCFHR1 (**Figure 3.8a**), suggesting at least two species of protein were being eluted in this process. To assess the purity and uniformity of the proteins in the various fractions the elution fractions were applied to a 10% SDS-PAGE gel and Coomassie stained. The data suggests the earlier peak (fractions 7-10) contains predominately non-specifically bound protein (**Figure 3.9a**), whilst the later peak (fractions 11-14) corresponds to the elution of rCFHR1. Fraction 11 also contains some higher molecular weight contaminant proteins so were excluded from further analysis. Fractions 12-14 were pooled, buffer exchanged, filter-concentrated, and the final concentration determined as outlined below (**Section 3.3.7**)

In the remaining clone (rCFHR3, rCFHR4B and rCFHR5), a single absorbance peak is observed on each UV trace of the eluted fractions (**Figure 3.9b-d**). The Coomassie stained SDS-PAGE gels confirmed this single peak was predominately the rCFHR proteins of interest with low levels of contamination proteins present. Thus, from the rCFHR3 elution, fractions 3-6 were selected, from rCFHR4B, fractions 6-9, and from rCFHR5, fractions 3-6.

During the purification process samples were collected from each stage of the protocol to assess the progress on HisTag purification. These samples were

analysed *via* western blot (**Figure 3.10**). An anti-CFH mAb C18/3 (Santa Cruz) to detect rCFHR1, followed by secondary detection with an HRPO-conjugated sheep anti-mouse IgG pAb (**Figure 3.10a**). Sample 1 is positive for rCFHR1, indicating successful production of the protein in the tissue culture growth media. Sample 2 shows that when diluting the tissue culture SN 1/2 in '2x His Binding Buffer', the conformation of rCFHR1 is not affected (e.g. no aggregation products detected). In sample 3, there is no evidence of rCFHR1, suggesting all detectable rCFHR1 is bound to the column. This is further confirmed by the positive result from sample 4, containing the eluted rCFHR1 protein. Samples 5 is a post-column growth media sample, and is negative for rCFHR1. This again confirms all detectable rCFHR1 has been purified.

In reviewing the western blot data for rCFHR1, identical conclusions can also be drawn from the data for rCFHR5 (**Figure 3.10d**); all available rCFHR5 protein is purified from the growth media (probing with an anti-CFH pAb). However, the data for rCFHR3 (**Figure 3.10b**) and rCFHR4B (**Figure 3.10c**) suggests there is an excess of recombinant protein still available in post-column sample 5. Further clarification comes from the positive result in sample 3 (pre-elute wash), for both proteins.

The success of this method, (i.e. high yield and low levels of non-specific protein contamination) indicated that additional purification steps such as Gel Filtration and Ion Exchange chromatography were not necessary.

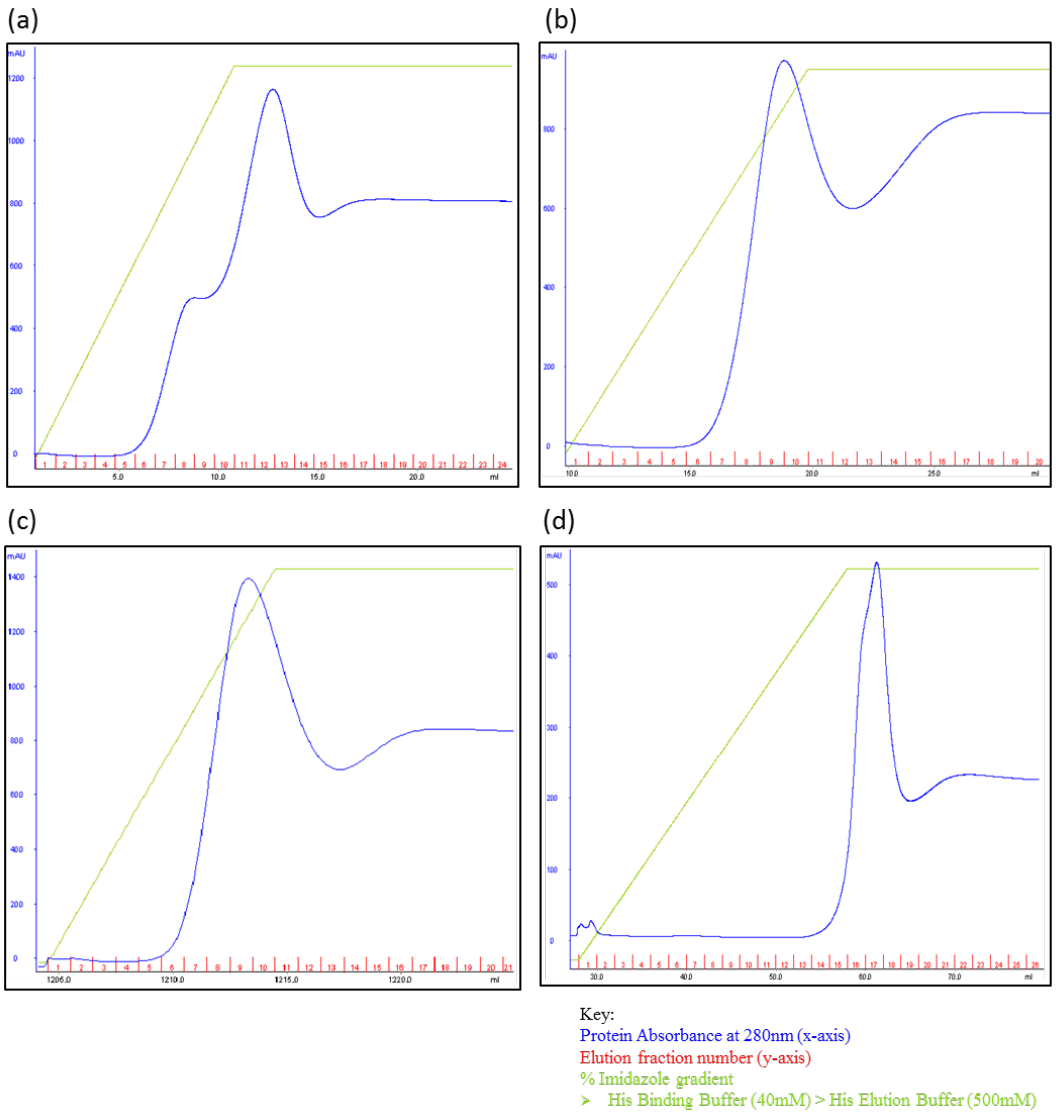


Figure 3.8: HisTag purification of rCFHR proteins

CHO/rCFHR tissue culture SNs were applied to a nickel-coated 5ml HiTrap™ Chelating column. Shown is the read out or trace of A280 nm (blue line), % imidazole in the gradient elution (green line) and the elution fraction number (denoted in red) from a representative HisTag purification using the ÄKTA prime (GE Healthcare) for **(a)** rCFHR1, **(b)** rCFHR3, **(c)** rCFHR4B and **(d)** rCFHR5.

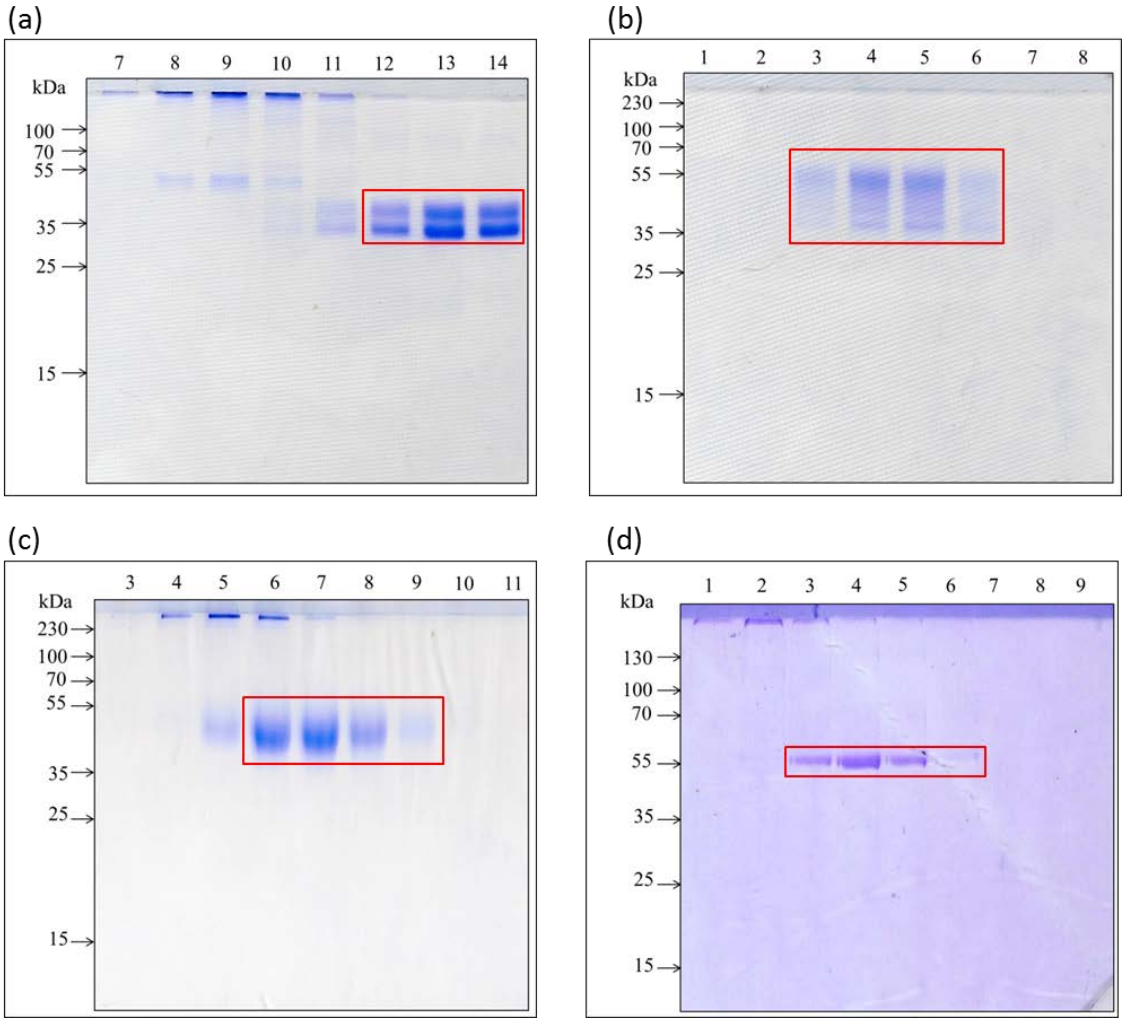


Figure 3.9: SDS-PAGE analysis of elution fractions from HisTag purifications

Fractions from the imidazole elution of each rCFHR protein were subjected to SDS-PAGE (12% gels). **(a)** rCFHR1, **(b)** rCFHR3, **(c)** rCFHR4B, and **(d)** rCFHR5. Gels were subsequent staining with Coomassie blue. Red boxes indicate the fractions which were pooled and buffer exchanged into PBS/NaN_3 0.01%. Data is representative of several purifications.

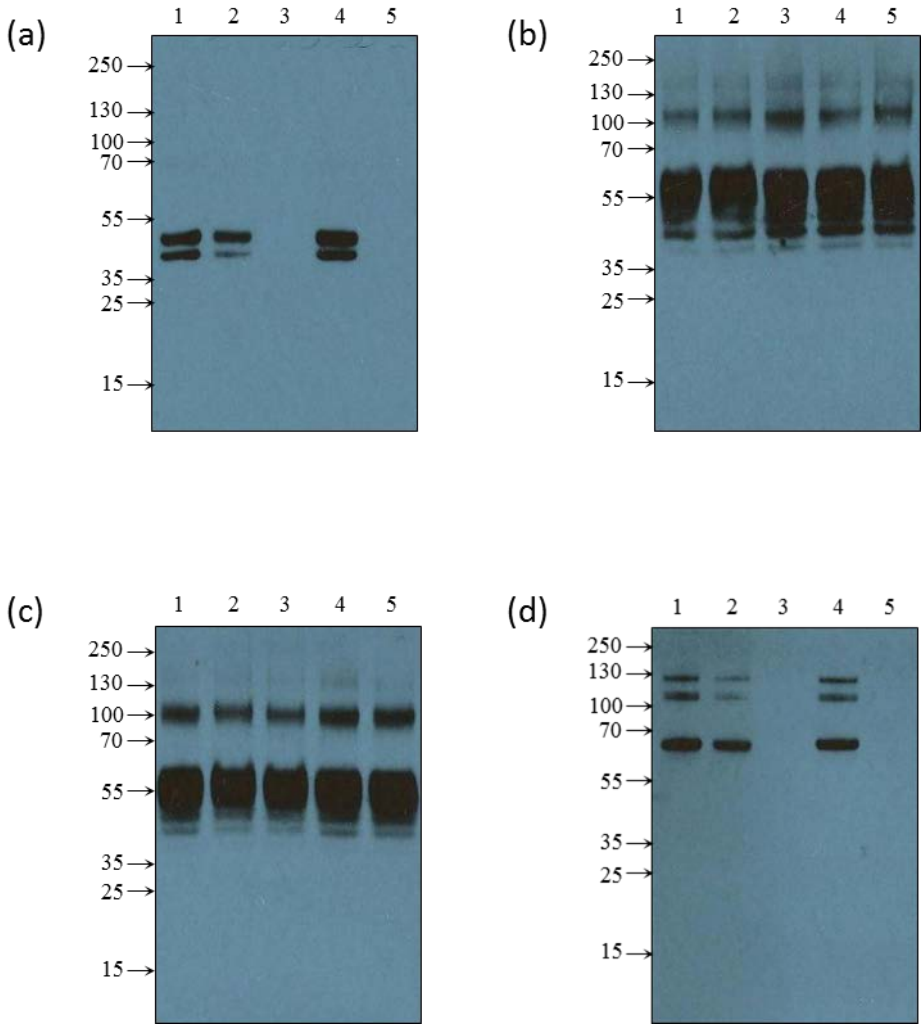


Figure 3.10: Detection of rCFHR proteins *via* western blot

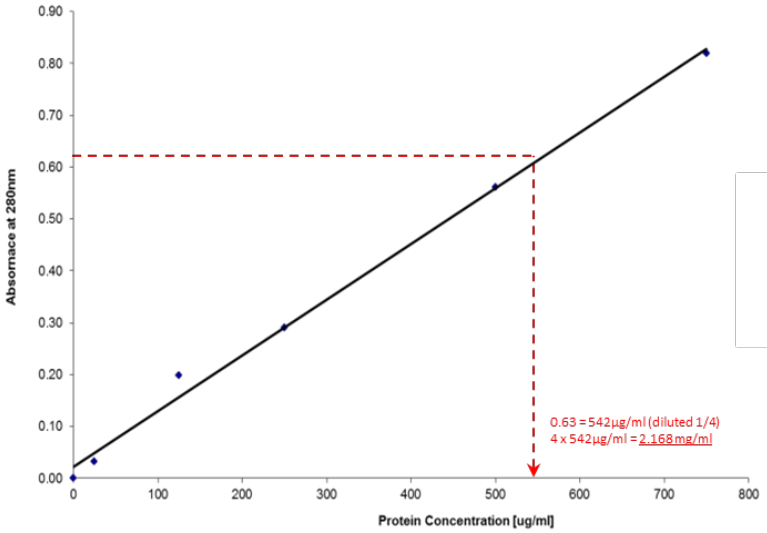
Samples collected at each stage of the HisTag purification procedure were subjected SDS-PAGE (10% gels) and transferred to a nitrocellulose membrane. **(a)** rCFHR1, **(b)** rCFHR3, **(c)** rCFHR4B, and **(d)** rCFHR5. After blocking with PBS/milk (5%), the nitrocellulose was probed with **(a)** L20/3 (1/2,000 dilution) followed by HRPO-conjugated anti-mouse IgG polyclonal (1/10,000 dilution) and **(b-d)** anti-CFH pAb (1/5,000 dilution) followed by HRPO-conjugated anti-goat IgG (1/10,000 dilution). Sample 1: CHO/rCFHR growth media (pre-column). Sample 2: 1/2 dilution of sample 1, diluted with '2x His Binding Buffer'. Sample 3: pre-elution PBS column wash. Sample 4: a sample taken from the most concentrated elution fraction. Sample 5: CHO/rCFHR growth media after column run (post-column).

3.3.7 Determination of rCFHR protein concentration

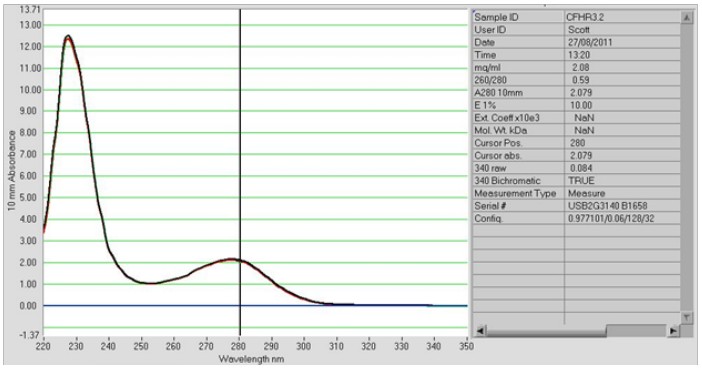
The selected rCFHR elution fractions were pooled, buffer exchanged into PBS/0.01% NaN_3 (pH 7.4), and concentrated to a volume of 0.5-1 ml (10,000MWCO Vivaspin20, Sartorius). The rCFHR3-5 proteins were successfully buffer exchanged and concentrated without any observable negative effects on protein composition. However, the rCFHR1 protein showed evidence of precipitation in response to buffer exchange and/or the effect of being concentrated. The removal of this precipitate was important for downstream work involving the induction and detection of Abs (**Chapters 4 and 5**). Aggregated or precipitated rCFHR proteins are highly likely to result in unique epitopes, which could result in an unwanted antigenic response (i.e. false positive results). Therefore, while removal of aggregates was necessary, it meant losing a portion of the purified rCFHR1. To remove the protein aggregates, the sample was centrifuged, followed by 0.2 µM filtration (0.2 µM Vivaspin6, Sartorius).

rCFHR protein concentration was determined *via* BCA Protein Assay (Thermo Scientific) and UV spectrophotometry (NanoDrop™ 8000, Thermo Scientific). The final protein concentrations were measured as follows: rCFHR1 at 0.2 mg/ml, rCFHR3 at 2.1 mg/ml (**Figure 3.11**), rCFHR4 at 3.4, and rCFHR5 at 0.5 mg/ml.

(a)



(b)



rCFHR3.2 Reading	A_{280} (10mm)	Conc. [mg/ml]
1st	2.104	2.10
2nd	2.052	2.05
3rd	2.079	2.08
Mean =	2.08	2.08

Figure 3.11: Determination of rCFHR protein concentration

(a) shows the 'line of best fit' plot from the BSA standard curve generated using a 'BCA Protein Assay' kit (Thermo Scientific) following manufacturer's guidance. The red dashed line denotes the intersect from the reading generated by 1/4 diluted rCFHR3 (clone rCFHR3.2) sample in this assay. **(b)** A representative UV spectrum is shown for the rCFHR3.2 protein as noted by spectrophotometry using the NanoDrop™ 8000. In-triplicate rCFHR3 A280 nm reads (refer to table) were taken compared to the protein storage buffer (PBS/NaN_3 0.01%, pH 7.4) and internal software used to calculate protein concentration (being set at 1 absorbance unit = 1mg/ml).

3.4 Discussion

The overall aims of this chapter were to produce a full set of rCFHR proteins. With the exception of rCFHR2, these aims were achieved. I have successfully cloned, expressed and purified sufficient quantities of rCFHR1, rCFHR3, rCFHR4B and rCFHR5 using a mammalian protein expression system.

Construction of the rCFHR2 protein expression plasmid was successful, and hygromycin B resistant DNA transfected CHO cell clones were selected. However, there was a difficulty in detecting, or confirming the presence of rCFHR2 protein in tissue culture growth media. As previously discussed, this is likely to be the result of a lack of cross-reactivity between the anti-CFH pAb and CFHR2. In previous western blot analysis of human sera (the only source of CFHR2 at the time of the experiment) and this polyclonal, there was no evidence of cross-reactivity with CFHR2. However, the native serum CFHR2 concentration is unknown so a poor performance *via* western blot may have been due to low levels of antigen availability. As previously described, western blot analysis using the anti-HisTag mAb failed to detect rCFHR2 or the other rCFHR proteins in the panel.

If the problem was related to an issue with rCFHR2 protein expression, it is important to consider which factors may be involved. For all *CFHR* cDNA clones, the native secretion signal peptides were maintained for expression in the CHO cell system. The native *CFHR2* signal peptide is identical to the sequence for *CFHR1* (UniProtKB Database; CFHR1: Q03591; CFHR2: P36980; amino acids 1-18: MWLLVSVILISRISSVGG), and the rCFHR1 protein was successfully produced. Therefore, a problem with rCFHR2 expression is unlikely to be related to an issue with signal sequence incompatibility with the CHO cell expression system. In addition, the same forward primer was used for cloning rCFHR1 and rCFHR2, so the recombinant Kozak[201] sequences for both mRNA transcripts would be identical. The remaining expression technology was contained in the *pDEF(L5H6)* expression vector, which was ubiquitously utilised by all *rCFHR* constructs.

In consideration of post-translational issues related to intracellular rCFHR2 processing, it is more difficult to hypothesise where problems may have arisen.

In light of the difficulties presented with rCFHR2 expression, it highlights the importance of adopting a more robust expression screening protocol that can be used to detect the expression of any recombinant protein (regardless of the availability of Abs for downstream protein detection). The use of reporter gene technology would be a useful addition to the current CHO cell/*pDEF(L5H6)* expression system. For example, the insertion of a GFP sequence upstream of the signal peptide is a viable consideration[202]. This would allow rapid identification of CHO cell transfectant colonies actively expressing the recombinant protein of interest. However, this particular methodology could only report on intracellular expression; it cannot provide confirmation protein secretion into the extracellular tissue culture media. Human CFHR2 has been successfully produced recombinantly in non-mammalian eukaryotic systems[203], so production in a mammalian system should be equally as suitable an expression environment, if not more so.

The CHO/rCFHR2 transfectant clones were cryogenically frozen and stored in LN_2 until a suitable detection reagent became available that could provide a positive control detection signal.

The experience with rCFHR2 illustrates the importance of both a good detection method and a specific means to purify the protein when generating novel recombinant proteins. From the outset, several key decisions had to be taken. These included the choice of expression vector, in concert with a suitable expression system (e.g. bacteria, yeast, insect, mammalian), the purification methodology (e.g. affinity, ion-exchange, PEG cuts, gel filtration, heparin). We chose to include a HisTag to the C-terminus of the rCFHR proteins to enable rapid purification *via* a nickel coated metal chelate column because it is a rapid method for purifying protein from a mixed batch of other proteins (i.e. FCS containing tissue culture media). The imidazole gradient elution in particular serves to resolve out non-specifically bound proteins based upon their relative affinity for the nickel surface, compared to that of the HisTag interaction. This method of purification has resulted in a single-step purification procedure, leading to the production of relatively pure recombinant protein stocks.

Using the HisTag has also served in providing an additional antigenic epitope that was utilised in detection screening of rCFHR1, rCFHR3, rCFHR4B and

rCFHR5 (**Figure 3.6**). However, as previously described, this failed to detect rCFHR2.

It was decided to incorporate the HisTag directly into the *pDEF(nMCS)* MCS, as this was deemed more efficient than inducing the HisTag into the initial PCR primer design. To insert the *L5H6*, a careful review of the available restriction enzyme sites led to the use of *BamHI*. The *BamHI* sequence (*GGATCC*) contains codons which translate to a glycine and serine, which made this a suitable candidate for linking the HisTag to each rCFHR protein. The amino acids have simple, non-reactive side-chains so often used for linker regions in recombinant fusion proteins. Another 3*glycine residues were added downstream of the *BamHI* contribution, followed by the 6*histidines and an introduced recombinant stop codon. Linker regions are often used in recombinant fusion proteins, and in particular, a linker made up of amino acids that will maintain a primary protein structure due to their small, non-reactive side chains[204]. In producing these linker-HisTags in this way, the aim was to ensure the HisTag and terminal SCR domain of each rCFHR protein were spaced sufficiently far apart that the chance of steric hindrance would be reduced with respect to either the HisTag, or the terminal SCR functionality.

Sequence analysis of all the rCFHR cDNA sequences revealed several single nucleotide polymorphisms or changes, but the most notable sequence variant was in CFHR4B. A Ser79Leu change was identified at the end of SCR1, which has the potential to affect the native hinge-region between SCR1 and SCR2. There is currently no data available regarding mutations or SNPs within the coding region of *CFHR4*, so it is unclear whether this change is a natural variant within the population, or a mutation introduced through bacterial cloning (before the cDNA came into our possession). I chose to proceed and produce this protein with this variant as there was no evidence suggesting it may cause downstream difficulties. It is possible that the amino acid change may confer a modification in the native structure and/or function of the protein. Production of the CFHR4 with a Leu at position 79, should also be carried out to assess whether there are any notable functional differences between these variants. All future work with the rCFHR4 Ser79Leu must be cautiously regarded, particularly with respect to cell/protein binding or functional analysis. Recent

data suggests CFHR4 is involved in the modulation of C-reactive protein (CRP). However, the CRP-binding data does not make reference to the importance of this region in the structure or other functions of this protein[205].

The advantage of the inclusion of HisTag quickly became evident during the purification of the rCFHR proteins. In particular, rCFHR3-5 were rapidly purified from cell culture SN at relatively high yields and excellent purities. However, purification of rCFHR1 was problematic. When initially purifying rCFHR1, the 'His Binding Buffer' concentration was set at the commonly used concentration of 20 mM. SDS-PAGE gel results from this approach showed a high level of background binding with CFHR1 and there was no evidence of the characteristic double banding of rCFHR1[153,198,200]. Furthermore, western blotting of these samples using C18/3 as the primary Ab indicated that very little of the 'purified' protein was rCFHR1. The concentration of imidazole in the 'His Binding Buffer' was increased to 40 mM with the aim of reducing the high level of non-specific background binding. This approach simply increases the imidazole coating the nickel column matrix, and therefore reduces the effect of non-specific interactions of other proteins. Only the HisTag protein, with significant affinity for the nickel coated column matrix (rCFHR1 in this case) should have the ability to displace the imidazole from the nickel and remain bound. This change was sufficient to markedly improve protein purification, both with respect to yield and overall purity.

The purity of the rCFHR proteins was very important, especially considering how they would be utilised in the next stage of work. The proteins would be essentially used as immunogenic substrates in two strands of work; (1) the generation of novel mAbs, with a high level of specifically for the rCFHR protein with which they had been raised, and (2) to screen whole serum for the presence of anti-CFHR autoantibodies in a cohort of aHUS patients.

In conclusion, the successful production of rCFHR1, rCFHR3, rCFHR4B and rCFHR5 enabled progression to the next strand of research; the production of mAbs and pAbs to specifically target each rCFHR protein.

Chapter 4

4. CFHR mAb production and development of CFHR detection assays

4.1 Introduction

In aHUS patients with a complete deficiency of *CFHR3/1* or *CFHR1/4*, there is a 90% increased risk of developing an IgG CFH autoantibody response. This suggests that the plasma concentration of CFHR1, either alone or in combination with CFHR3 or CFHR4, has an impact of the development of a CFH-targeted autoantibody response. To assess the role CFHR concentration has in disease phenotype, a specific sensitive assay is required. A commonly used methodology for detecting protein concentration in a mixed protein sample is a sandwich ELISA. This requires the use of a capture and detection Ab, both of which target the CFHR protein of interest.

There are several commercially available CFH monoclonal and pAbs, but a restricted availability of Abs to specifically target CFHR proteins. CFH Abs are commonly used to detect CFHR proteins, by taking advantage of natural cross-reactivity of this highly homologous protein family[159,162,203]. However, using ELISA to measure CFHR concentration in human sera requires at least one Ab specific to the CFHR protein of interest. Therefore, to measure the individual concentration of each CFHR protein in human sera, production of CFHR-specific mAbs was a requirement.

Deficiency of CFHR1, CFHR3 and CFHR4 have previously been implicated in conferring an increased risk of developing CFH autoantibodies. Therefore, CFHR-specific ELISA tests would be developed against these proteins.

CFHR5 had also been implicated in disease, so testing CFHR5 levels was also an important piece of work. However, there was already a CFHR5-specific commercially available mAb available that could be used for this work (R&D Systems, UK; MAB3845).

4.2 Chapter 4 Methodology: Hybridoma Technology

4.2.1 Discovery of Hybridoma Technology

The first discovery of Abs of a defined specificity began with the discovery that mAbs are produced in fairly high quantities in multiple myelomas, a disease caused by the cancerous growth of a B-cell clone that secretes Abs[206-208]. In the 1960s, Potter induced myelomas in mice by administering intraperitoneal injections of mineral oil[209]. Although these myeloma Igs produced were monoclonal and long-lived, their antigenic specificity was unknown. In contrast, immunised mice produced sera of known antigenic reactivity, yet the sera was a polyclonal mixture of Abs and the B-cells were short-lived *in vitro*. Milstein and Kohler combined the advantageous properties of these two types of Ab-secreting cells and revolutionised immunology with the development of hybridoma technology[210]. They fused the non-secretor variants of the murine mineral-oil induced myelomas with antigen-primed B-cells *in vitro*, making immortalised Ab-secreting cells called 'hybridomas'.

4.2.2 Producing Hybridomas

Primed B-cells are obtained from experimental animals (usually mice, rats or hamsters) immunised with the designated antigen. The myeloma cells that the B-cells are fused with are mutant cell lines that fail to secrete their own Igs and carry a selectable trait. After fusion, the cells are grown in a culture media that allows the growth of the hybridomas, but inhibits the growth of the unfused myeloma that carry the selectable trait. Hybridomas are then cloned and the specificity of their Abs is established an antigen-binding assay. Finally, the hybridoma cells generating the Ab of the desired specificity are cloned and expanded.

4.2.3 Myeloma Fusion Partner Cell Lines

Two myeloma variants that have defects in nucleotide synthesis pathways are commonly used as fusion partners. One of these lacks the gene for *hypoxanthine guanine phosphoribosyl transferase* (*HGPRT*), an enzyme that is essential in the salvage pathway of purine synthesis (adenosine monophosphate, AMP; guanosine monophosphate, GMP). Under normal

conditions, myelomas can continue to make purines by the alternative *de novo* pathway.

The other myeloma variant lacks the thymidine kinase (*Tk*) gene that is required for making thymidylate from thymidine. *Tk* variants can usually still synthesise thymidylate from uridylate *de novo*. Following fusion, the cells are grown in a culture medium containing a supplement known as 'HAT', containing hypoxanthine, aminopterin and thymidine. Aminopterin is a dihydrofolate analog that blocks the reactivation of tetrahydrofolate, which is critical for the *de novo* synthesis of GMP, AMP and thymidine monophosphate (TMP). Cells grown in HAT supplemented media that have *HGPRT* and *Tk* genes can survive since exogenous hypoxanthine and thymidine are supplied in the media. If a myeloma fusion partner lacking one of these genes fuses with a B-cell, the B-cell supplies the vital gene and the hybridoma will survive. However, unfused myelomas that are *HGPRT* or *Tk* deficient will die, as aminopterin blocks the *de novo* synthesis pathway and the cells are unable to utilise the salvage pathways.

To generate hybrimomas, experimental animals are immunised with the antigen of interest. Generally the mice are primed with an antigen that has been emulsified in an adjuvant (e.g. complete Freund's adjuvant) in order to enhance the immunogenicity of the antigen. After an appropriate period of time, their spleens (or lymph nodes) are removed and a cell suspension is made. Polyethelene glycol (PEG) allows the cell membranes of the antigen–specific B-cells and the non-secretor *HGPRT-/-* (or *Tk-/-*) myeloma fusion partners to fuse. After fusion, cells are grown in HAT supplemented media that contain aminopterin. Because aminopterin inhibits *de novo* purine synthesis, the *HGPRT-/-* (or *Tk-/-*) myeloma cells that must synthesise their nucleotides *de novo* will die off. Unfused B-cells (and other splenocytes) also die, despite their ability to synthesise purines in the presence of aminopterin, as their life-span is restricted when outside of their native environment. Hybridomas have inherited longevity from the myeloma cells and the ability to synthesise purines (or thymidylate) *via* the salvage pathways in the presence of aminopterin.

In a fusion, only 1% of the starting cells fuse, and only 0.001% form stable hybrids. The hybridoma cells are cloned under limiting-dilution conditions to guarantee the monoclonality of the Ab-producing cell.

4.2.4 Hybridoma Screening

Various techniques have been used to find and select the hybridoma producing the desired Ab. Assays commonly used to find and select the hybridoma producing the desired Ab. Assays commonly used include ELISA, radioimmunoassay (RIA), immunofluorescence, cytotoxicity assays, and immunoprecipitation. Simple procedures have been worked out to screen hybridomas for both the Ab specificity and class. The class of the Ab is particularly important if using the mAb in partnership with a secondary Ab which must have specificity to the isotype and species of the monoclonal. Cloned hybridoma cells are grown in *in vitro* culture or in murine ascites to yield virtually unlimited amounts of a mAb. The concentration of mAb in culture SN in approximately 1-100 µg/ml, verse 1 mg/ml in ascites[211].

The selected screening protocol is summarised in Chaper 2 (**Figure 2.3**).

4.3 Chapter Aims

The aim of this chapter was to produce specific mAbs against CFHR1, CFHR3 and CFHR4, to be used to develop a CFHR-specific detection ELISA and measure protein concentration in healthy and disease sera.

4.4 Results

4.4.1 Preliminary Hybridoma Production and Screening

To optimise and gain valuable experience in hybridoma production, several initial attempts were made at producing mAb-secreting hybridomas, prior to the recombinant production of CFHR1, CFHR3 and CFHR4B. Therefore, truncated CFH fragments were used to immunise mice with the aim of targeting an Ab response to CFH SCR domains 19-20 (fragment: $rCFH_{19-20}$), and CFHR1 domains 4-5 (fragment: $rCFHR1_{4-5}$). Between these fragments there are only 2 amino acids difference; S1191L and V1197A changes located in CFH SCR 20, and CFHR1 SCR 5. The main aim of choosing these fragments was to try and raise Abs to the amino acids variants, thus raising either a CFH-specific mAb, with specificity for the SCR 20, or a CFHR1-specific mAb with specificity for SCR 5. Both potential Abs could then be used to build highly specific detection ELISA screens.

Two groups of mice were immunised with each antigen, and assuming a successful adaptive immune response, both groups of mice should produce Abs that cross-react with both antigens, and also produce variant specific Abs.

After incubating the hydridoma fusions in HAT supplemented selection media for 10 d, tissue culture SN was collected and tested *via* direct ELISA (**Section 2.3.4.1**) for the presence of antigen-specific IgG Abs; 960 wells were screened on antigen coated ELISA plates. To determine a threshold for positivity, the population mean plus two standard deviations (mean+2SD) was applied to the data. Detection results that were above, around, and below the threshold were considered, positive, borderline positive, and negative, respectively. ELISA plates were coated with equimolar concentrations of $rCFH_{19-20}$ and $rCFHR1_{4-5}$, and SN from the $rCFH_{4-5}$ hybridomas loaded into coated wells. The results from each antigen screen were aligned, $rCFH_{19-20}$ in blue and $rCFHR1_{4-5}$ in red (**Figure 4.1**).

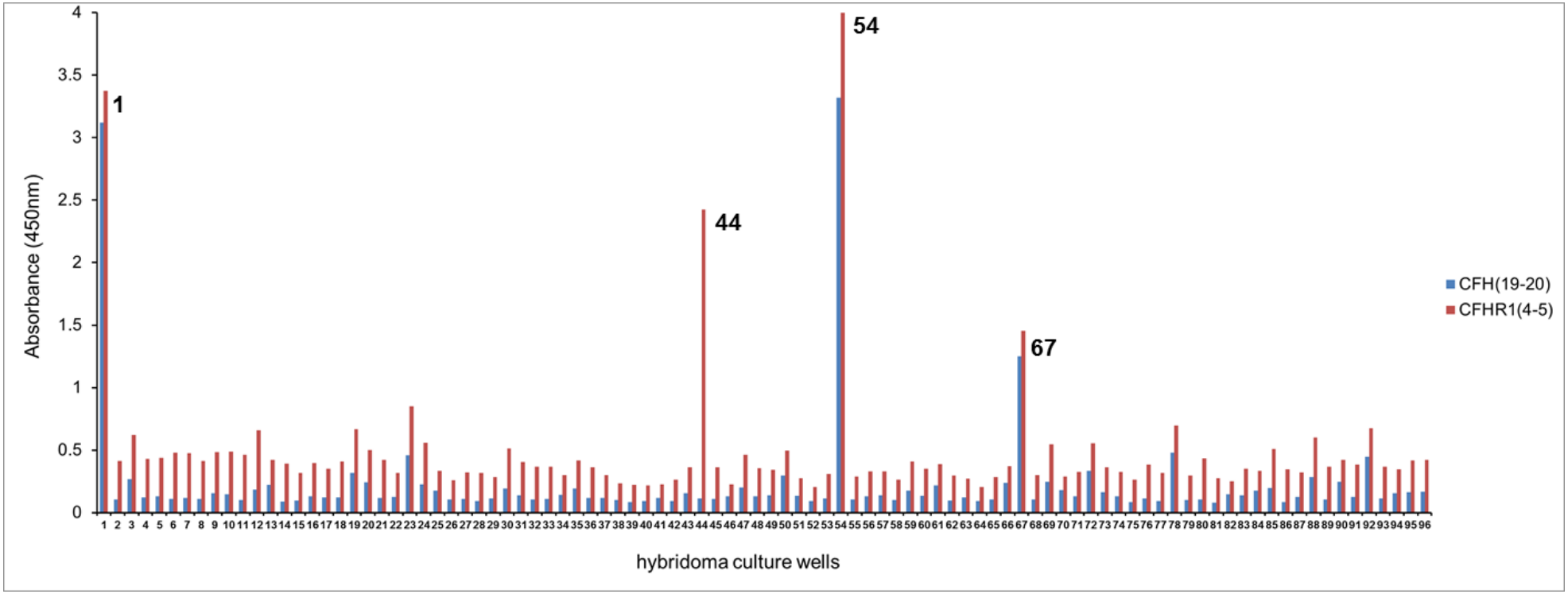


Figure 4.1: ELISA screen of rCFHR1$_{4-5}$ hybridoma SN

After incubation the post-fusion hybridomas for 10 d, 96 wells containing hybridoma colonies were selected and transferred to a single 96 well plate. After incubating for 3 d, culture SN was collected and applied to an ELISA plate coated with equimolar concentrations of rCFH$_{19-20}$ and rCFHR1$_{4-5}$ to screen for IgG Ab production. Bound murine IgG was detected using an HRPO-conjugated goat anti-mouse IgG. Each hybridoma well SN was screened against both antigen fragments, and results aligned for comparison. rCFH$_{19-20}$ is shown in red, and rCFHR1$_{4-5}$.

The results show 4 positive results, 3 with cross-reactivity for both proteins (hybridoma wells 1, 54, and 67), and one with specificity for rCFHR1$_{4-5}$ only (well 44). These hybridoma wells were selected and subjected to a limiting dilution to isolate monoclonal populations of Ab producing hybridomas. The cultures were re-screened as before, and positive wells were carried forward. However, once the final positive clones had been selected and carried forward to large batch culturing, Protein G purification failed to purify IgG from the tissue culture SN, despite a positive signal from the ELISA screen.

Upon investigation, it was discovered the secondary Ab being used was manufactured to detect murine IgG, but the sample had not been adsorbed to IgG resulting in potential cross-reactivity with other immunoglobulin isotypes. From the remaining positive clones, tissue culture SN was tested using a murine IgG-specific secondary, alongside an IgM-specific secondary. The results indicated that all hybridomas were producing IgM Abs (**Figure 4.2**). Subsequent to this discovery, all remaining hybridomas either ceased secreting Abs, or were lost due to cell death.

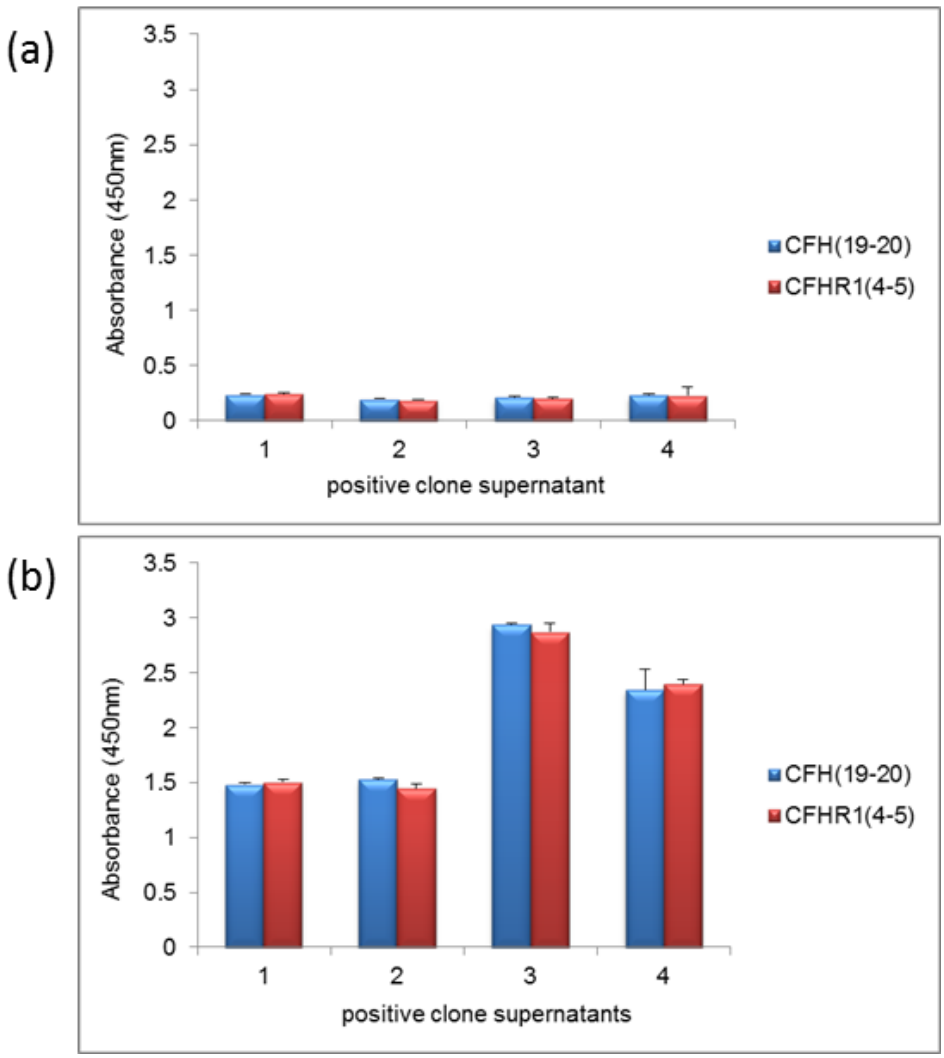


Figure 4.2: CFHR1$_{4-5}$ hybridoma screening reveals IgM subclass

SN from 4 rCFHR1$_{4-5}$ hybridoma clones (1-4) was screened on rCFH$_{19-20}$ and rCFHR1$_{4-5}$ coated ELISA plates, with subsequent detection using, **(a)** HRPO-conjugated goat anti-mouse IgG, and **(b)** HRPO-conjugated goat anti-mouse IgM.

From this first round of hybridoma production and screening, the two main issues that arose were production of an IgM response rather than IgG, and the loss of Ab secretion by selected hybridoma cultures. Hybridoma cell death is a routine part of hybridoma production[212]. It was decided that the antigen load per immunisation was not enough to drive an IgG immune response, so after a literature review[210,213,214] the antigen load for the next round of immunisations was increased to 40 µg per immunisation.

The next attempt at producing Ab-secreting hybridomas was completed using a different antigen. The immunising antigen was changed because previous fragments were no longer available at the levels required for the newly optimised immunisation protocol. Therefore, a fragment consisting of CFH SCR domains 10-12 ($rCFH_{10-12}$) was selected. When comparing all CFH family proteins for SCR sequence homology, this region has the lowest level of homology with SCR domains of the CFHR proteins (CFHR1-4, in particular). However, the region does share ~60% homology with CFHR5 SCR domains 3-5, so there is still potential for cross-reactivity at least one other CFH family protein. If we are to consider the remaining non-homologous sequence between the two regions of CFH and CFHR5, there is a ~40% chance of producing a CFH-specific response.

The splenocytes were collected from CFH_{10-12} challenged mice, and hybridoma fusions completed, as before (**Section 2.4.2**). Previously collected tail bleeds were screened against $rCFH_{10-12}$ and an Ab response was assessed. The results indicate that when immunising mice with 40 µg $rCFH_{10-12}$ per immunisation, an IgG response is raised against $rCFH_{10-12}$ (**Figure 4.3**).

Hybridomas were screened as previously described (**Section 2.3.4.1** and **Figure 2.3**), and initial screening results identified 3 positives results (hybridoma wells: 16, 17 and 24), and 3 borderline positives (**Figure 4.4; 1st screen**). The second round of screening on these cultures showed hybridoma clones 17 and 24 maintaining positivity (**Figure 4.4; 2nd screen**). However, in clone 16 the level of positivity reduced to a borderline result. The third round of screening (before selecting individual monoclonal hybridomas) shows clones 16, 17 and 24 maintaining a positive result, while other selected clones continued to test negative (**Figure 4.4; 3rd screen**). Positive clones 16, 17 and

24 were subjected to a limiting dilution, as previously described (**Figure 2.3**) to select individual CFH_{10-12}-specific hybridomas. However, all positive clones failed to grow under limiting-dilution conditions. The original 'stock' cultures also failed to grow after being transferred to fresh media (**Figure 4.4; 4th screen**). Therefore, all CFH_{10-12} hybridoma clones were eventually lost.

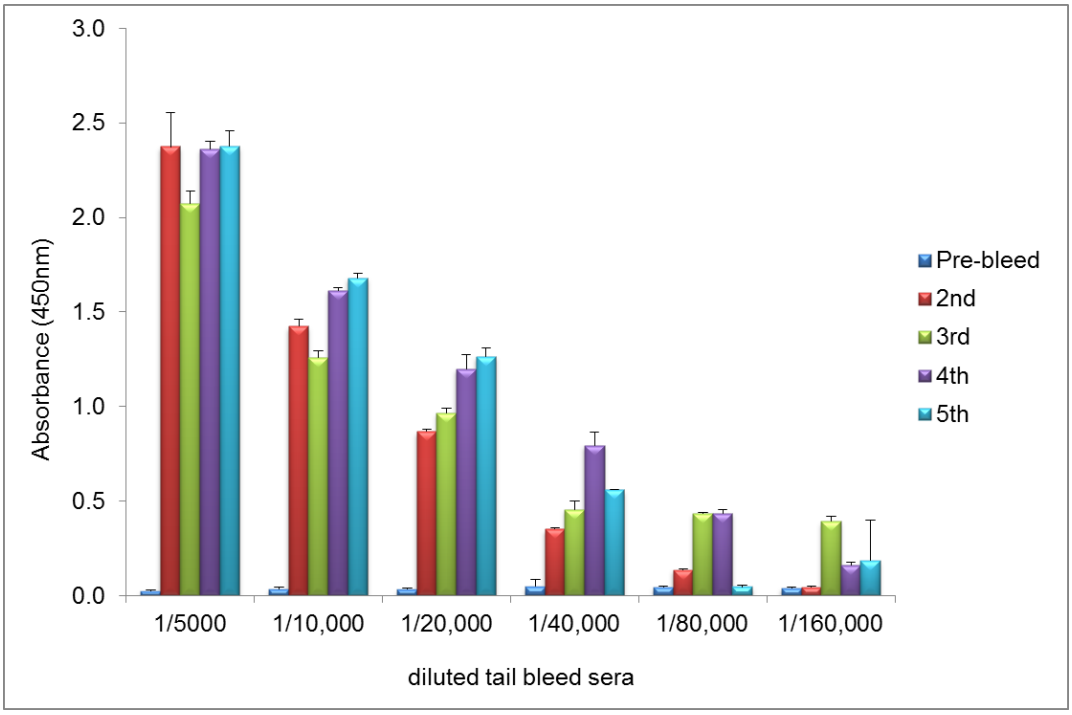


Figure 4.3: IgG response detected in CFH_{10-12} tail bleed

C57BL/6 mice were immunised fortnightly over 8 weeks. Prior to each immunisation, a tail bleed was taken. Sera was collected and diluted to 1/5000 in BSA blocking solution (1% BSA/PBS, pH 7.4), followed by a 1/2 serial dilution (1/5,000-160,000). A 96-well ELISA plate was coated with 100ng CFH_{10-12} per well and diluted murine sera was applied in-triplicate to the CFH_{10-12} coated wells. After blocking the plate with BSA blocking buffer, an HRPO-conjugated goat anti-mouse IgG secondary Ab was applied. Pre-bleed: d +0 (blue), 2nd: d +14 (red). 3rd: d +28 (green), 4th: d +42 (purple), 5th: d +56 (cyan).

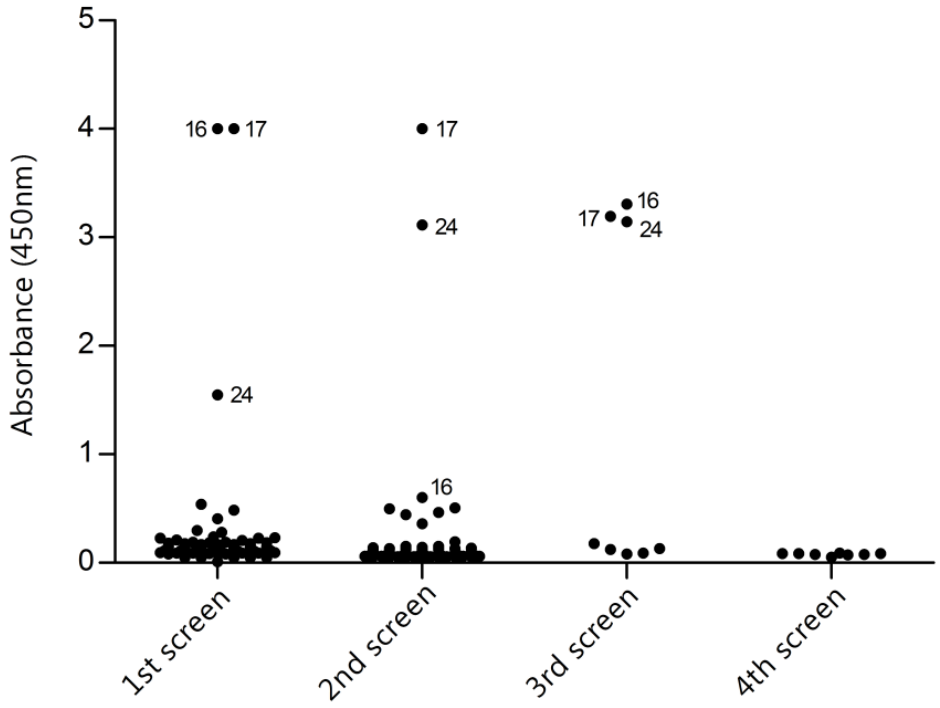


Figure 4.4: CFH_{10-12} hybridoma screening

After incubation the post-fusion hybridomas for 10 d, 84 wells containing hybridoma colonies were selected and transferred to a single 96 well plate. After incubating for 3 d, culture SN was collected and applied to an ELISA plate coated with 100ng $rCFH_{10-12}$ to screen for IgG Ab production. Bound murine IgG was detected using an HRPO-conjugated sheep anti-mouse IgG. The graph shows 4 rounds of screening, highlighting the progression of viable hybridomas, with emphasis placed on 3 consistently positive clones (16, 17 and 24).

4.4.2 Detection of rCFHR1 Ab response from murine tail bleeds

After successful production and purification of rCFHR1, mice were immunised using the optimised protocol, as previously described (**Section 2.4.1.1**). Tail bleeds taken from mice immunised with rCFHR1 indicate a positive IgG anti-rCFHR1 response after each immunisation (**Figure 4.5**).

The results from rCFHR1 hydridoma production will be presented as representative of this methodology. Therefore, rCFHR4B hybridoma production and screening results will not be shown, but will be commented upon. However, results for testing of all mAbs will be shown.

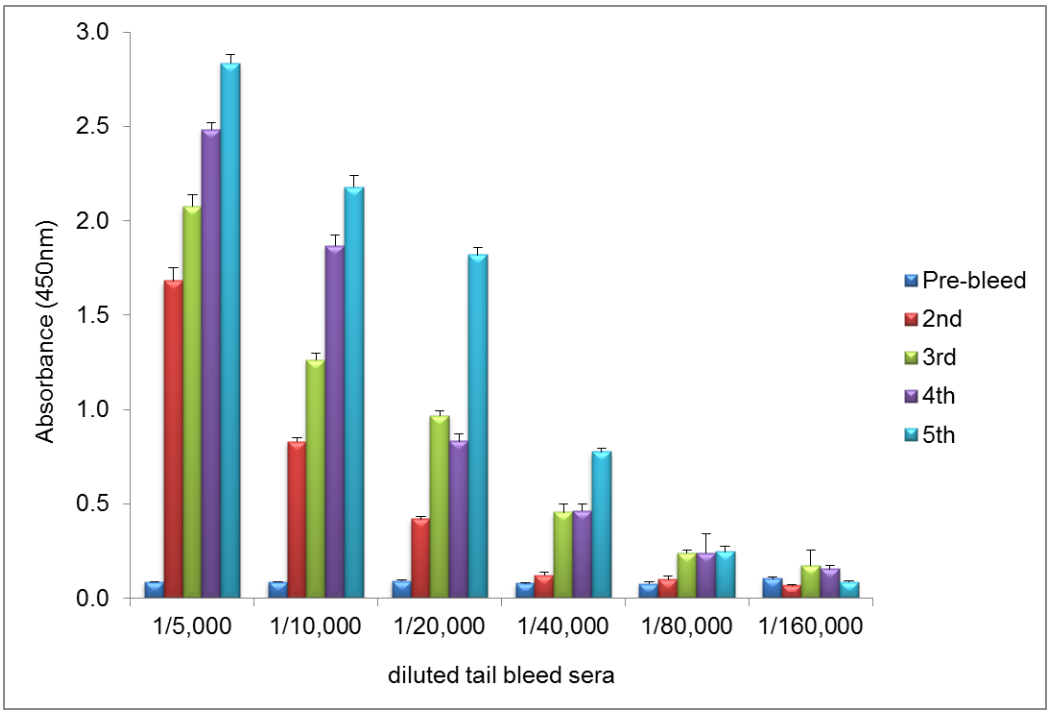


Figure 4.5: Detection of rCFHR1 Ab response from murine tail bleeds

C57BL/6 mice were immunised fortnightly over 8 weeks. Prior to each immunisation, a tail bleed was taken. Sera was collected and diluted to 1/5000 in BSA blocking solution (PBS/BSA 1%, pH 7.4), followed by a 1/2 serial dilution (x-axis; 1/5,000-160,000). A 96-well ELISA plate was coated with 100 ng rCFHR1 per well. The diluted murine sera was applied in-triplicate to the rCFHR1 coated wells, followed by detection of bound IgG with an HRPO-conjugated goat anti-mouse IgG secondary Ab. Pre-bleed: d +0 (blue), 2nd: d +14 (red). 3rd: d +28 (green), 4th: d +42 (purple), 5th: d +56 (cyan).

4.4.3 Hybridoma Screening: rCFHR1

The first round of screening was completed on all 960 hybridoma fusion wells (10 x 96 well plates). Previously, the first round of hybridoma screening was completed only on wells with evidence of hybridoma colony formation. The SN from these wells was discarded, and colonies condensed from 10 x 96-well plates, to a single plate. However, due to a change in the hybridoma fusion protocol (increasing fusion partner cell count to 10^7 and using all splenocytes), the number of hybridomas colonies also increased. On each plate, there was on average 80% of wells with colony growth, with approximately 1-3 colonies per well. From previous experience, screening SN from the initial fusion cultures leads to a high level of background signal. However, due to the high volume of resulting hybridomas, it was deemed essential to screen all culture wells. As expected, the first round of screening yielded a high level of background signal. The previous method for creating a threshold for positivity was applied to these results (i.e. mean+2SD), resulting in the selection of 74 hybridoma culture wells with a positive or borderline positive detection signal (**Figure 4.6**).

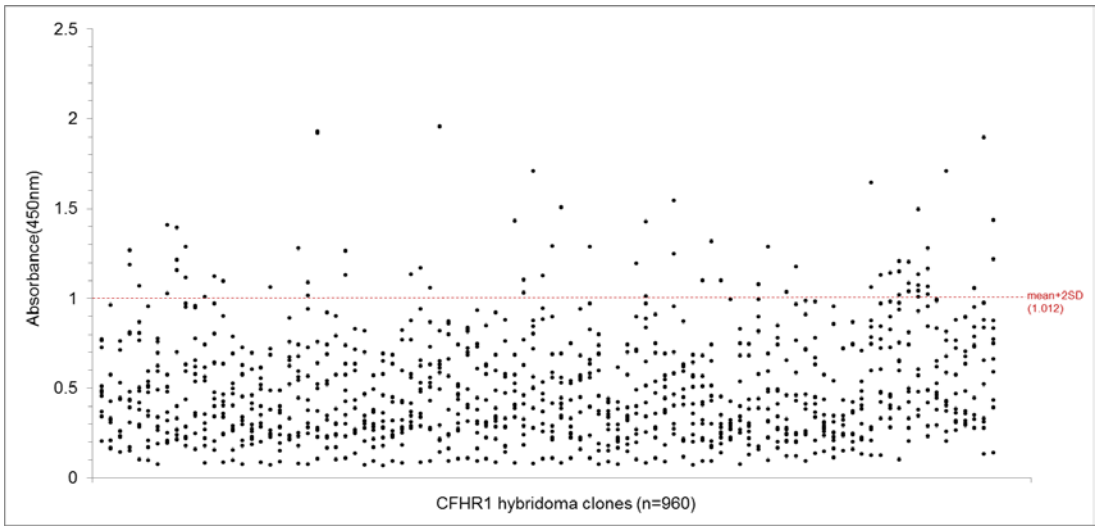


Figure 4.6: ELISA Screen of rCFHR1 Hybridomas (1st screen)

960 hybridoma fusion wells were screened for the presence of rCFHR1-specific mouse IgG Abs. Plates were coated with 100ng rCFHR1 per well, followed by the addition of hybridoma well SN. Bound murine IgG was detected using an HRPO-conjugated sheep anti-mouse IgG. The results from the hybridoma cohort were used to calculate a threshold between a positive and negative result (indicted by the red dashed line; mean+2SD). 74 positive wells were selected as positive and carried forward for further testing.

The 74 selected hybridoma cultures were transferred to fresh media and condensed to a single 96-well plate. The second round of screening was completed after culturing for 4 d, and included antigen screening for rCFHR1, native CFH and a HisTag control protein (**Figure 4.7**). There were 7 observable positive results (7, 12, 18, 27, 63, 67, and 70) from the 74 hybridomas screened, all of which tested positive for the rCFHR1 antigen. Clones 7 and 63 appear to exhibit cross-reactivity with CFH, and clones 12, 18, 67 and 70 appear to bind to rCFHR1 exclusively (when compared to the antigens tested in this particular screen). Clone 18 appears to cross-react with the HisTag control, suggesting this culture well contains an anti-HisTag producing hybridoma. The HisTag control protein is a CFH fragment consisting of SCR domains 16-18, so there is potential for cross-reactivity between rCFHR1 and $rCFH_{16\text{-}18}$, especially between rCFHR1 SCR domain 3, and rCFH domain 18, as they share 100% amino acid sequence homology. However, native CFH serves as a negative control protein in this analysis; there are no positive clones with cross-reactivity for both native CFH and $rCFH_{16\text{-}18}$. Additionally, native CFH does not have a recombinant HisTag, and therefore serves as a negative control for HisTag cross-reactivity in this ELISA analysis.

After a further round of screening these positive controls, only hybridoma clones 18, 67 and 70 survived and maintained positivity (**Figure 4.8**). However, only clone 70 survived to be taken forward to the stages of monoclonal selection *via* limiting dilution. The final hybridoma clone selected came from clone rCFHR1.10.37, resulting in the mAb being named ‘R1/1037’.

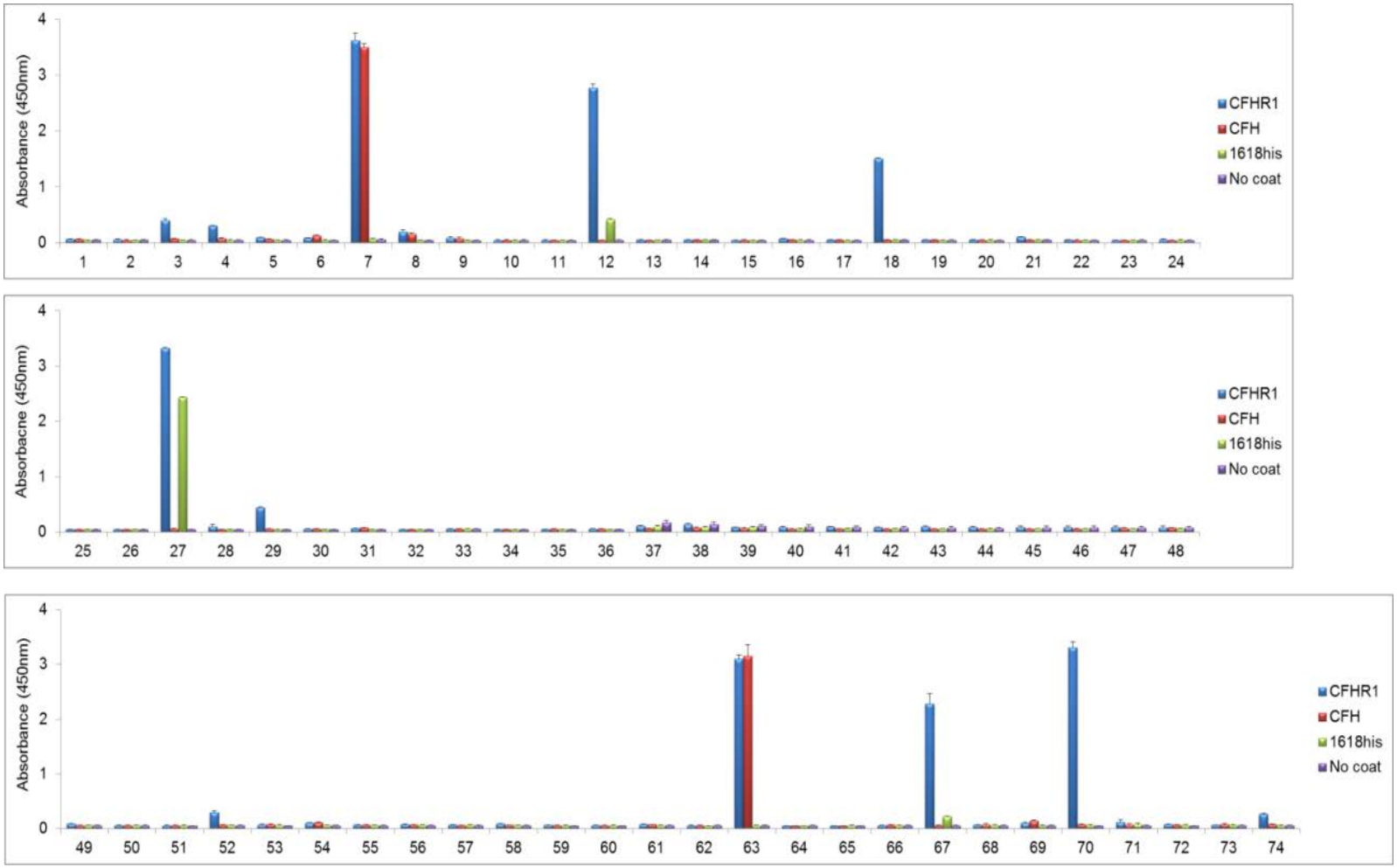


Figure 4.7: ELISA Screen of rCFHR1 Hybridomas (2nd screen)

The selected 74 positive hybridoma wells (1-74) were resuspended into 500 µl HAT-supplemented tissue culture media and cultured for 7 d. Tissue culture SN was then screened against rCFHR1, CFH and 1618his (HisTag negative control) as before.

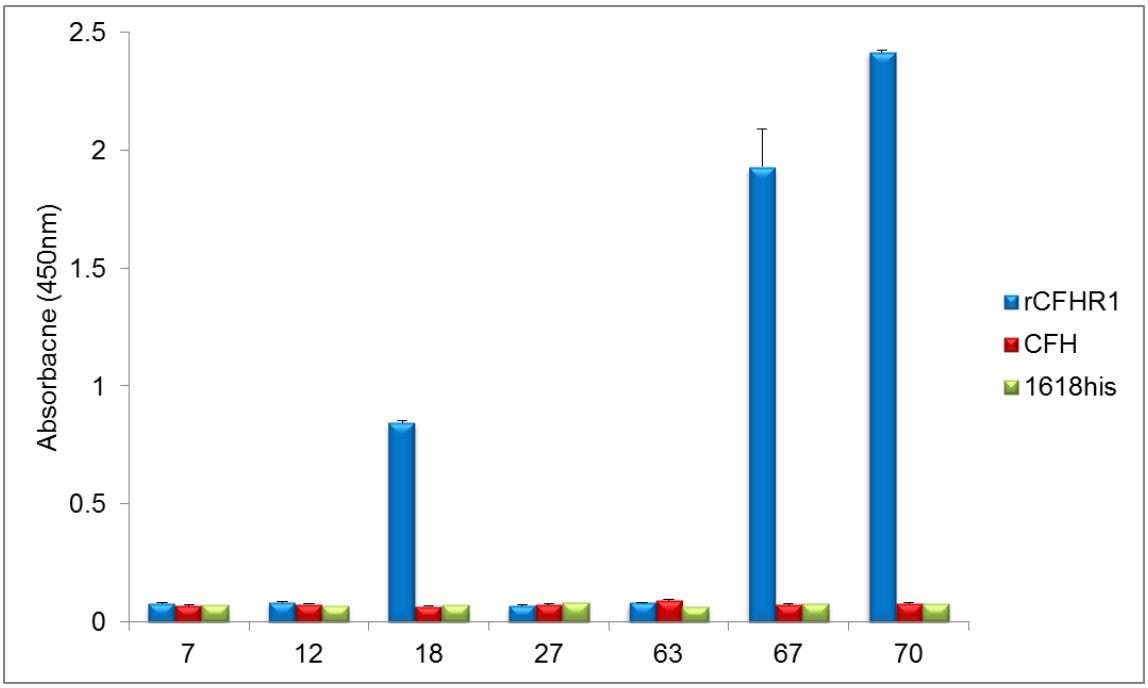


Figure 4.8: ELISA Screen of rCFHR1 Hybridomas (3rd screen)

Positive clones 7, 12, 18, 27, 63, 67 and 70 were carried forward from the previous screen, re-cultured for 3 d and screened as before. Clones 18, 67 and 70 were selected as positive.

The same methodology was used to screen for CFHR4B hybridomas, but using CFHRB coated plates to screen. 64 positive clones were identified after the 1st screen, 12 of which were selected for the 2nd screen. After the 3rd and final screen, three viable positive clones remained, referred to as R4/244, R4/277 and R4/123.

4.4.4 Purification of mAbs R1/1037 and R4/244

All selected hybridoma clones were transferred to individual large batch cultures, and after 10 d 300 ml of tissue culture SN was collected. The purification results of R1/1037 and R4/244 will be now be shown. The R1/1037 SN was prepared as previously described (**Chapter 2**) and ran through a Protein G coated chromatography column to purify murine IgG Abs. After running, the column was eluted using a Glycine-HCl (pH 2.4) acid elution, followed by neutralisation of eluate fractions with 1 M Tris base (pH 11.0). The elution was completed using the AKTA prime system, with partnered Unicorn software. The results show a protein absorbance peak across fractions 1-4 (**Figure 4.9a**). Elution fractions were reduced and separated on 10% SDS-PAGE gels, followed by Coomassie staining as previously described (**Chapter 2**). The results of the Coomassie stain show the characteristic heavy and light chains of a reduced murine IgG (**Figure 4.9b**). These samples were selected, pooled, buffer exchanged and concentrated using Vivaspin 20 (30,000MW)

R4/244 was purified using the same methodology (**Figure 4.10**).

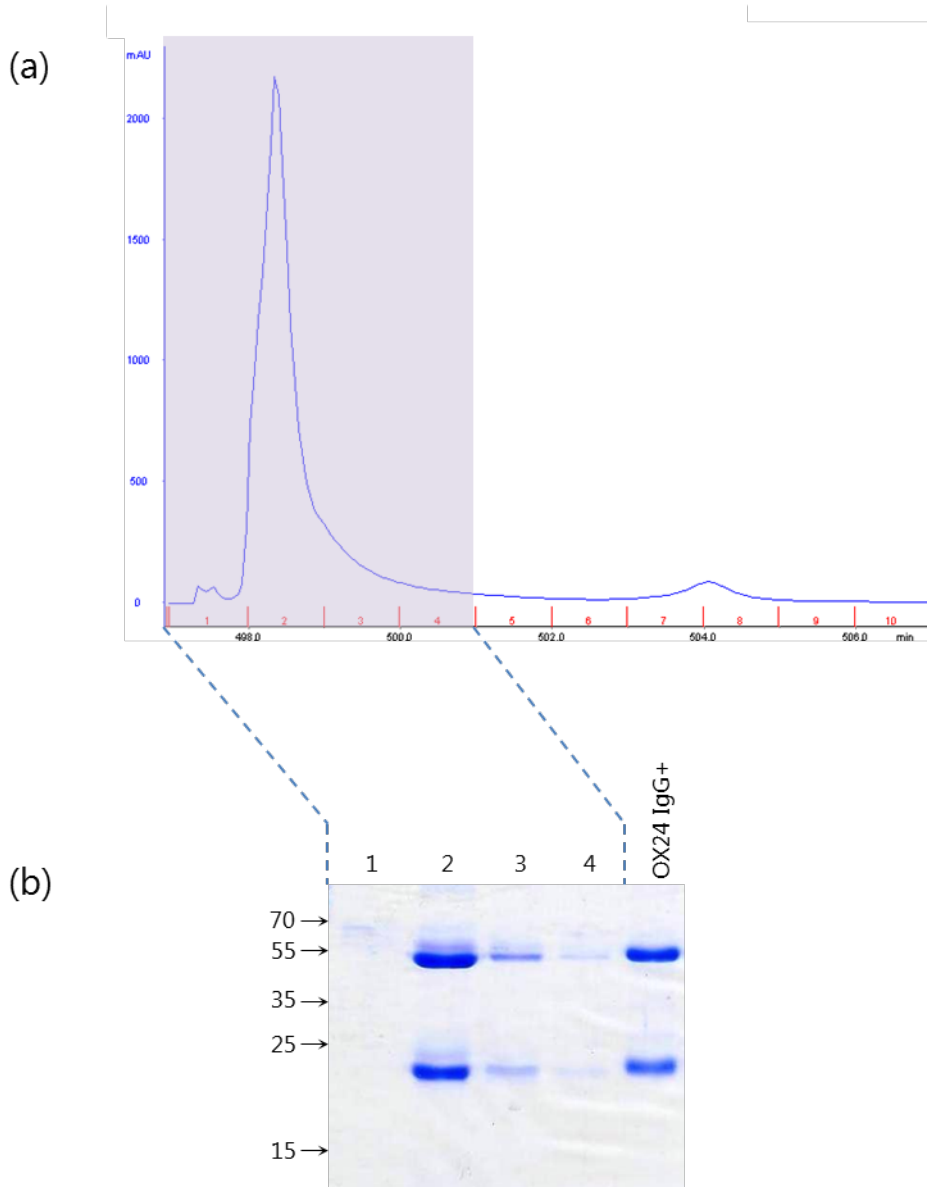


Figure 4.9: Purification of R1/1037 mAb

The R1/1037 hybridoma clone was cultured for 10 d, and 300ml of the culture SN was diluted 1/2 with PBS (adjusted to pH 7.4). Diluted SN ran over a 1ml Protein G affinity chromatography column, followed by a PBS (pH 7.4) wash, then a Glycine-HCl (pH 2.5) acid elution. **(a)** Chromatogram of the Glycine-HCl elution. The x-axis shows 1ml elution fractions (red; 1-10). The y-axis shows protein absorbance at 280 nm. **(b)** Fractions 1-4 were reduced (to heavy and light chains) and electrophoresed on a 10% SDS-PAGE gel, followed by staining with Coomassie blue. A reduced IgG positive control mAb was loaded into the final well (OX24 IgG+).

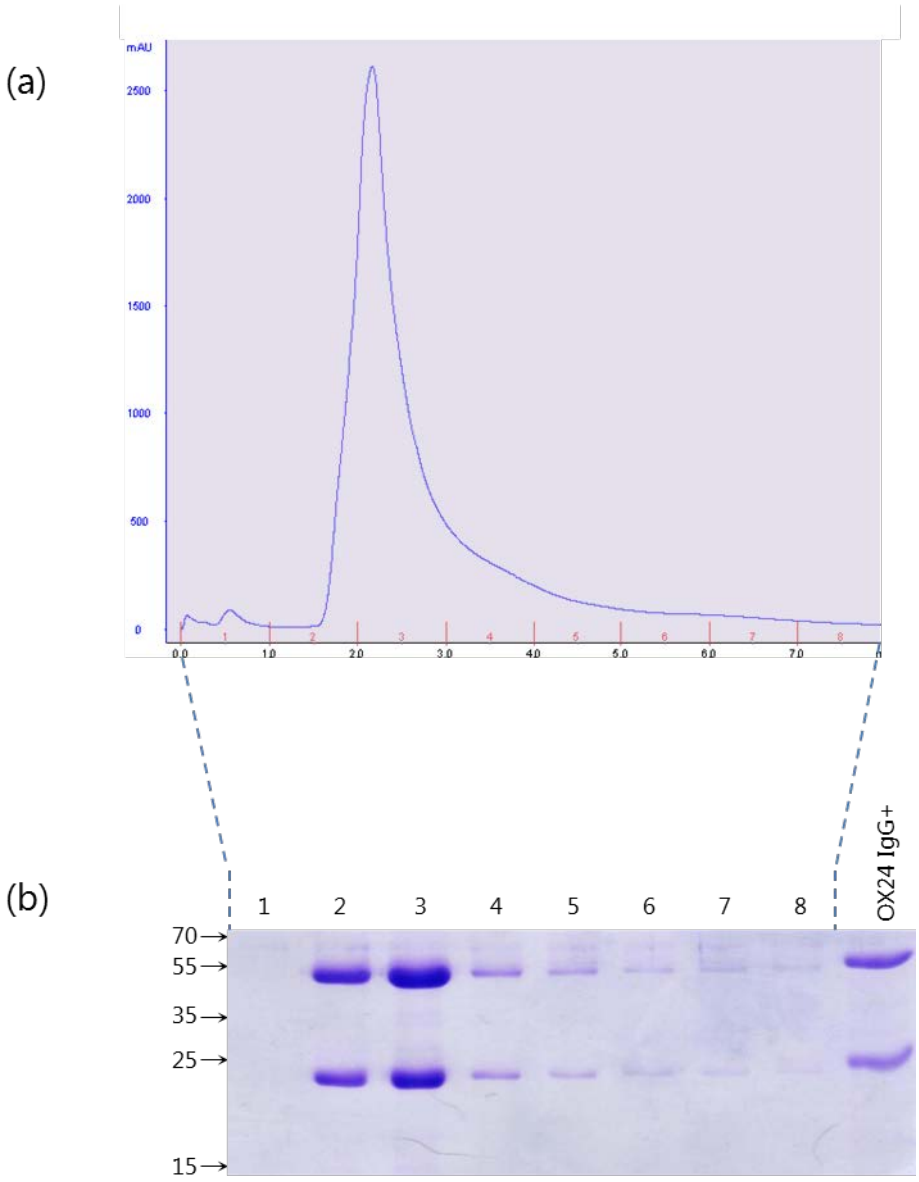


Figure 4.10: Purification of R4/244 mAb

The R4/244 hybridoma clone was cultured for 10 d, and 300ml of the culture SN was diluted 1/2 with PBS (adjusted to pH 7.4). Diluted SN ran over a 1ml Protein G affinity chromatography column, followed by a PBS (pH 7.4) wash, then a Glycine-HCl (pH 2.5) acid elution. **(a)** Chromatogram of the Glycine-HCl elution. The x-axis shows 1ml elution fractions (red; 1-10). The y-axis shows protein absorbance at 280 nm. **(b)** Fractions 1-8 were reduced (to heavy and light chains) and electrophoresed on a 10% SDS-PAGE gel, followed by staining with Coomassie blue. A reduced IgG positive control mAb was loaded into the final well (OX24 IgG+).

4.4.5 Ab Isotype Testing of R1/1037, R4/244, R4/277 and R4/123

To further clarify the R1/1037 heavy and light chain isotypes, R1/1037 and R4/244 were coated to ELISA plates and screened using HRPO-conjugated anti-murine IgG1, $IgG2_a$, $IgG2_b$, IgG3, IgM and IgA detection Abs, as previously described (**Chapter 2**). The light-chains were also determined using HRPO-conjugated anti-murine kappa and lambda light chain detection Abs. The results show R1/1037 and R4/244 to both have an IgG1 heavy chain, and kappa light chain (**Figure 4.11**). OX24 was included as a positive control for IgG1 and kappa.

4.4.6 Cross-reactivity testing of R1/1037, R4/244, R4/277 and R4/123

The hybridoma screening highlighted R1/1037 as having rCFHR1-specificity, when cross-reactivity tested against CFH and a HisTag control. However, it was unclear whether this mAb cross-reacted with CFHR2, CFHR3, CFHR4 or CFHR5. The rCFHR proteins produced in Chapter 3, could now be utilised for cross-reactivity screening.

ELISA plates were coated with equimolar concentration of native CFH, rCFHR1, rCFHR3, rCFHR4B, rCFHR5 and the HisTag control protein ($rCFH_{16\text{-}18}$). R1/1037, R4/244, R4/277 and R4/123 were all screened across all coated antigens. The results indicate R1/1037 binds exclusively to rCFHR1, with no evidence of cross-reactivity with the other coated CFH family proteins (**Figure 12**). R4/244 and R4/277 bind exclusively to rCFHR4B. However, R4/123 binds to rCFHRB, but also cross-reacts with CFH. These data do not include rCFHR2 screening because of the protein production issues, as previously described (**Chapter 3**).

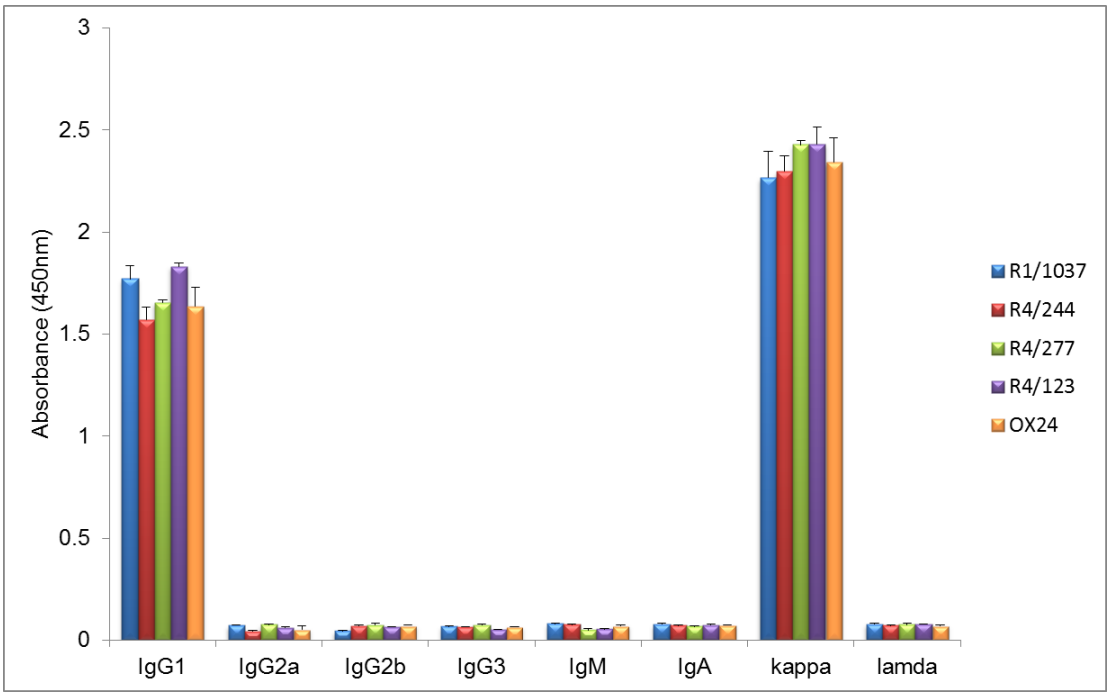


Figure 4.11: Ab isotype testing R1/1037, R4/244, R4/277 and R4/123

ELISA plates were coated in-triplicate with equal concentrations R1/1037, R4/244, R4/277, R4/123 and OX24 (IgG1 positive control). Isotypes were detected using HRPO-conjugated Abs against murine heavy chains IgG1, $IgG2_a$, $IgG2_b$, IgG3, IgM and IgA. HRPO-conjugated Abs against murine kappa and lamda light chains were also detected.

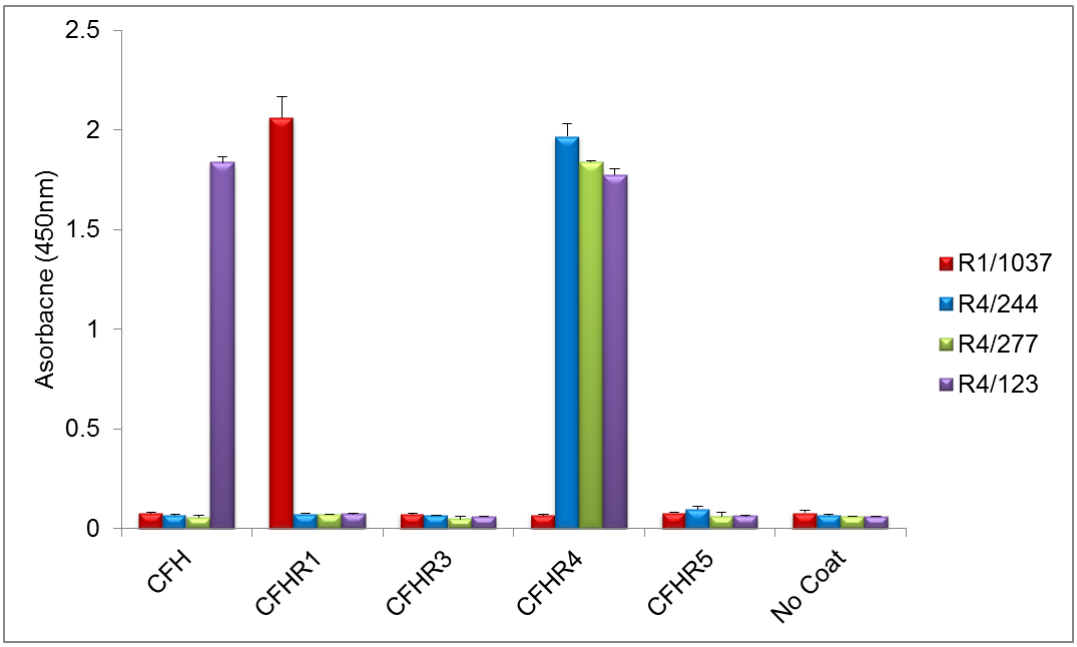


Figure 4.12: Specificity testing of R1/1037, R4/244, R4/277 and R4/123

ELISA plates were coated in-triplicate with equimolar concentrations of CFH, rCFHR1, rCFHR3, rCFHR4B and rCFHR5. R1/1037, R4/244, R4/277 and R4/123 were diluted 1/2000 and applied to each set of coated wells. Bound murine IgG was detected using a HRPO-conjugated sheep anti-mouse IgG secondary pAb.

4.4.7 Ab-specificity testing against native CFH family proteins

Due to the lack of purified CFHR2 for use as an ELISA coating antigen, human plasma was the only source of CFHR2. However, coating an ELISA plate with plasma was not a viable option due to the presence of native CFHR1. In addition, capturing CFHR2 was not possible due to the lack suitable Abs. Therefore, western blot analysis of human serum was used to assess the potential cross-reactivity of R1/1037 with CFHR2. This would enable separation of the CFH family proteins by size, and give CFHR1 and CFHR2 distinct and separate positions in the detection results.

Human serum was collected from *CFHR1-/-* aHUS patients to use as CFHR1 negative controls in the western blot analysis, in comparison to a positive control serum (*CFHR1*+/+). The CFHR2 status of these patients was unknown at the time of testing. When probing human serum *via* western blot, R1/1037 binds to CFHR1 in the positive control serum. However, in the *CFHR1* homozygous gene deficient aHUS patients, R1/1037 is not detecting CFHR1 (**Figure 4.13**). This confirms that R1/1037 can be used in western blot to detect native serum CFHR1. However, the results also indicated R1/1037 is cross-reacting with a lower molecular weight pair of proteins which sit either side of the 25 kDa protein marker. This is evident in all serum samples. This double banding is characteristic of two isotypes of CFHR2 found in human serum, suggesting R1/1037 cross-reacts with native CFHR2, and therefore is not CFHR1-specific.

The same methodology was used to screen R4/244, R4/277 and R4/123. The results show R4/244 binds exclusively to a protein with the region of CFHR4A (48 kDa), but fails to highlight the native CFHR4B (45 kDa) protein (**Figure 4.14a**). R4/123 binds the CFHR4A (86 kDa) band, but also cross-reacts with CFH (155 kDa) protein (**Figure 4.14b**). There is an additional band highlighted between MW markers 35-55 kDa. Patients 1-6 are CFHR3 deficient, therefore ruling out CFHR3 cross-reactivity. This protein is either CFHR4B (45 kDa) or CFHL-1 (47 kDa). As CFHL-1 lacks N-linked glycosylation sites (Asn-X-Ser/Thr), the spread of this band is unlikely to be a non-glycosylated protein. This suggests the band to be CFHR4B, although there is absence of the protein in patient 1, and the control sample.

R4/277 was shown to bind CFHR4A exclusively (**Figure 4.14c**). However, in a subsequent repeat of this screen, R4/277 showed evidence of cross-reactivity with a lower MW band, assumed to be CFHR4B. As previously observed with R4/123 screening, patient 1 and the control serum were negative for this protein band. This suggests the same protein is being detected by both mAbs. It is currently unclear why the lower MW band signal intensity is so different between the R4/123 and R4/277 screens, especially as the CFHR4A signals appear similar. Further categorisation is required to confirm this protein is CFHR4B.

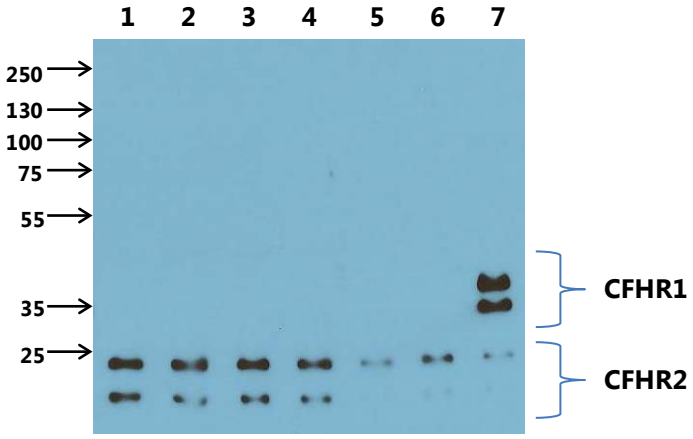


Figure 4.13: R1/1037 cross-reactivity with human serum proteins

Human serum was diluted 1/10 and electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% NFDM/PBS. This blot was stained with R1/1037 (diluted 1/5,000), followed by detection with HPRO-conjugated sheep anti-mouse IgG, diluted (1/10,000). Serum samples 1-6 are from aHUS patients with a *CFHR1* homozygous gene deficiency, and serum sample 7 is a BTS control with two copies of *CFHR1*.

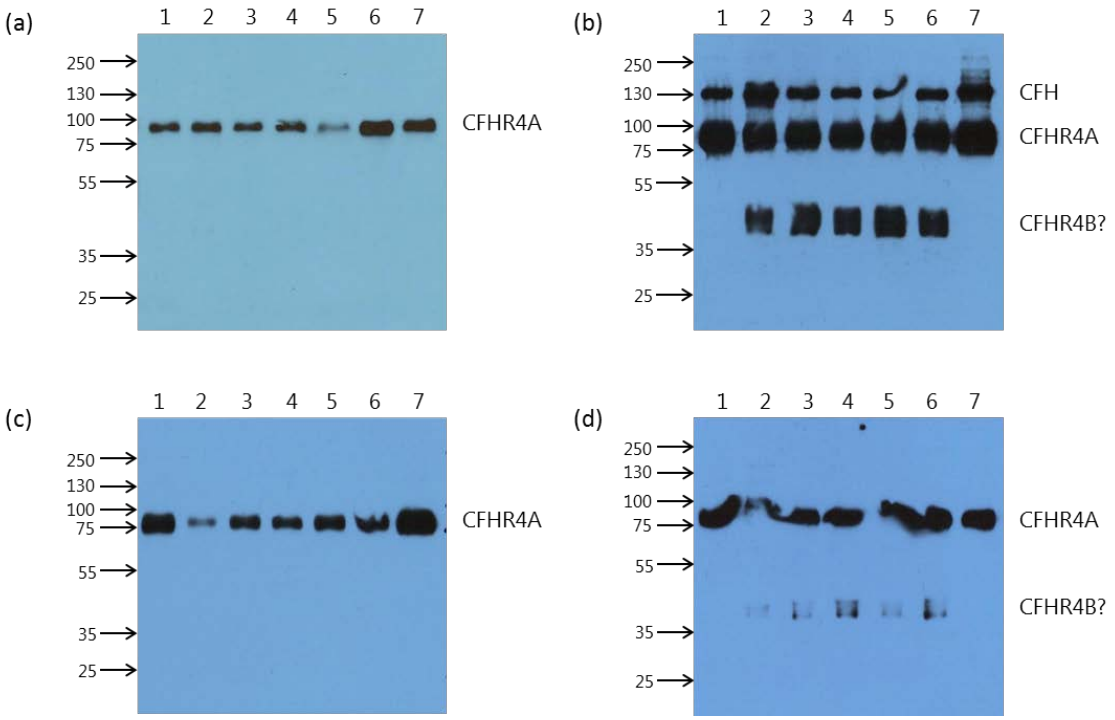


Figure 4.14: R4/244, R4/277 & R4/123 cross-reactivity with human sera

Human serum was diluted 1/10 and electrophoresed on 10% SDS-PAGE gels. Proteins were transferred to a nitrocellulose membrane and blocked with 5% NFDM/PBS. **(a)** This blot was stained with R4/244 (diluted 1/5,000) followed by detection with HPRO-conjugated sheep anti-mouse IgG (diluted 1/10,000). Serum samples 1-6 are from aHUS patients with a *CFHR3* homozygous gene deficiency, and serum sample 7 is a BTS control with two copies of *CFHR3*. **(b)** This blot was stained with R4/123 (diluted 1/5,000), followed by the same detection reagent as before. **(c)** This blot was stained with R4/277 (diluted 1/5,000), followed by the same detection reagent as before. **(d)** This is a repeat of **(c)**.

4.4.8 Purification of native CFHR1/CFHR2 from human sera

To further clarify the binding potential of R1/1037, the Ab was covalently coated to a HiTrap™ NHS-activated column (**Section 2.3.3**). Human serum was collected from a blood donor control, known to have a least one gene copy of each of *CFHR1, CFHR3* and *CFHR4*. Again, the CFHR2 status was unknown. 40 ml of serum was collected and diluted in 1/10 with PBS. The diluted serum solution was filter purified (0.2 µM) and the final pH confirmed as 7.4. After running through the column and washing with filter-sterilised PBS, R1/1037 bound serum proteins were eluted using an acid elution, as previously described in the Protein G purification protocol (**Section 2.3.2**). The elution was completed and monitored using the AKTA primer system and related Unicorn software.

Elution fractions were separated on a 10% SDS-PAGE gel, followed by Coomassie staining (**Figure 4.15**). The results show the characteristic double banding for CFHR1 and CFHR2[147,150]. There were other observable bands, again suggesting the R1/1037 mAb binds CFHR1, but also has cross-reactivity for CFHR2. These results also show R1/1037 binds to these proteins in their native form, as previous analysis *via* ELISA and western blot dictated that CFHR1 (*via* direct ELISA and western blot) and CFHR2 (*via* western blot only) were presented to R1/1037 in a potentially denatured form (coated directly to an ELISA plate, or treated with SDS and boiling).

Additionally, NHS-columns were made for R4/244, R4/277 and R4/123. However, CFHR4 mAb-coated columns failed to purify defined protein bands, and contained a high level of contamination. It is unclear why each CFHR mAb was unable to specifically purify native CFHR4.

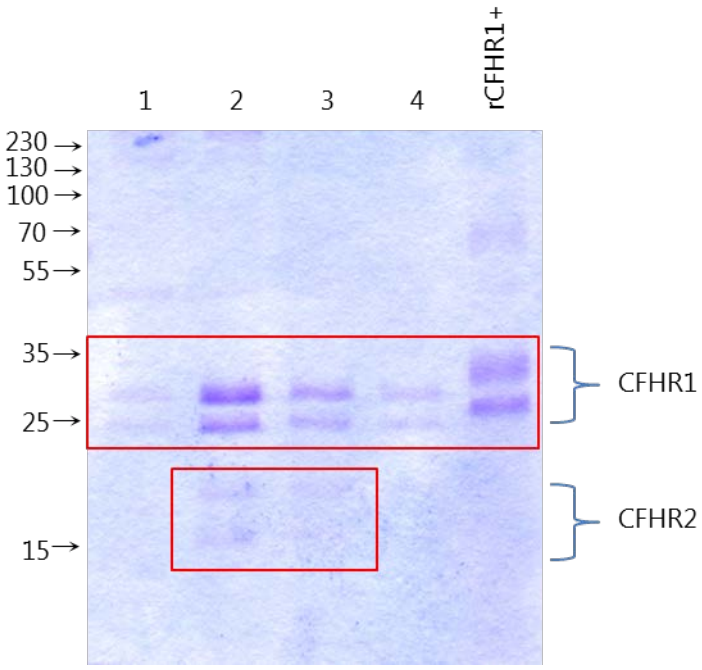


Figure 4.15: R1/1037 column purification of native CFHR1 and CFHR2

R1/1037 was covalently coated to a HiTrap NHS-activated column (GE Healthcare). 10ml human serum was diluted 1/10 in PBS/NaN_3 0.01% (pH 7.4) and run over the R1/1037 coated column. After washing with PBS, bound protein was eluted using 0.1 M glycine HCl (pH 2.5). Lanes 1-4 represent the corresponding elution fractions. Purified rCFHR1+ was loaded as a positive control.

4.4.9 Detection of rCFHR2 in CHO/rCFHR2 Tissue Culture SN

As R1/1037 cross-reacts with CFHR2, we now had a suitable Ab reagent to detect the recombinant production of CFHR2 in CHO/rCFHR2 cell tissue culture SN. A new transfection was completed using *pDEF/rCFHR2* and wildtype CHO cells, as previous described (**Section 2.2.2.1**). Post-transfection, 28 x CHO/rCFHR2 transfectant single clones were selected and tissue culture SN collected after 14 d of culturing from a single cell. Direct ELISA was still not an option for testing, but western blot offered potential due to the separation of protein (as previous described). After testing all 28 clones *via* western blot, R1/1037 was able to detect rCFHR2 production in all clones (**Figure 4.16** shows a sample of 7 of these clones). In all 28 clones tested, the western blot signals were assessed *via* densitometry, and the highest signal was deemed to be the clone producing rCFHR2 with most abundance. However, all clones were producing the protein at level <5%+/- the mean densitometry value of the clone population. Clone R2.2 was selected and carried forward for large batch culturing of rCFHR2.

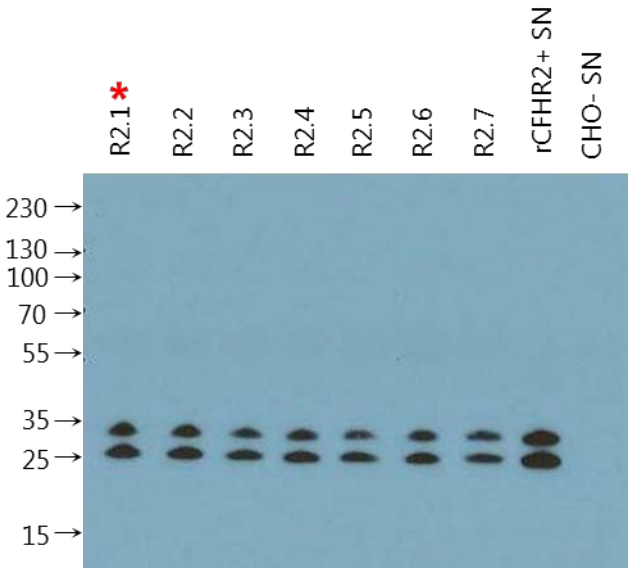


Figure 4.16: rCFHR2 detection in tissue culture SN

rCFHR2 tissue culture SN from 7 monoclonal cultures of CHO/rCFHR2 (clones R2.1-7) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was stained with R1/1037 at 1/2000 dilution, followed by detection with an HRPO-conjugated sheep anti-mouse IgG. Positive (rCFHR2+SN) and negative (CHO-SN) control tissue culture SN were also loaded. The clone with the highest signal (*) was selected and carried forward for large batch production.

4.4.10 Purification of rCFHR2 *via* HisTag purification

Clone R2.2 was cultured for 10 d, and 300 ml tissue cultures was collected and prepare as previously described for HisTag purification (**Section 2.3.1**). An imidazole concentration of 20 mM was selected as a starting concentration in the 'HisTag Binding Buffer' (as used for purification of rCFHR3, rCFHR4B and rCFHR5). The AKTA purification chromatogram shows elution of protein in fractions 6-17 (**Figure 4.17a**), spaced over two elution peaks, the latter of which is most likely rCFHR2.

Elution fractions were separated on 10% SDS-PAGE gels, followed by Coomassie staining as previously described (**Figure 17b**). The results of the Coomassie stain show non-specific binding of protein in fractions 7-12, and rCFHR2 in fractions 12-17. Fraction 12 appears to be the interchange between non-specifically bound proteins and rCFHR2. Therefore, fractions 13-17 were selected, pooled, buffer exchanged and concentrated using Vivaspin 20 (10,000MW) columns (Sartorius).

This completes the panel of rCFHR proteins required to undertake the autoantibody screening of aHUS and IgAN patients.

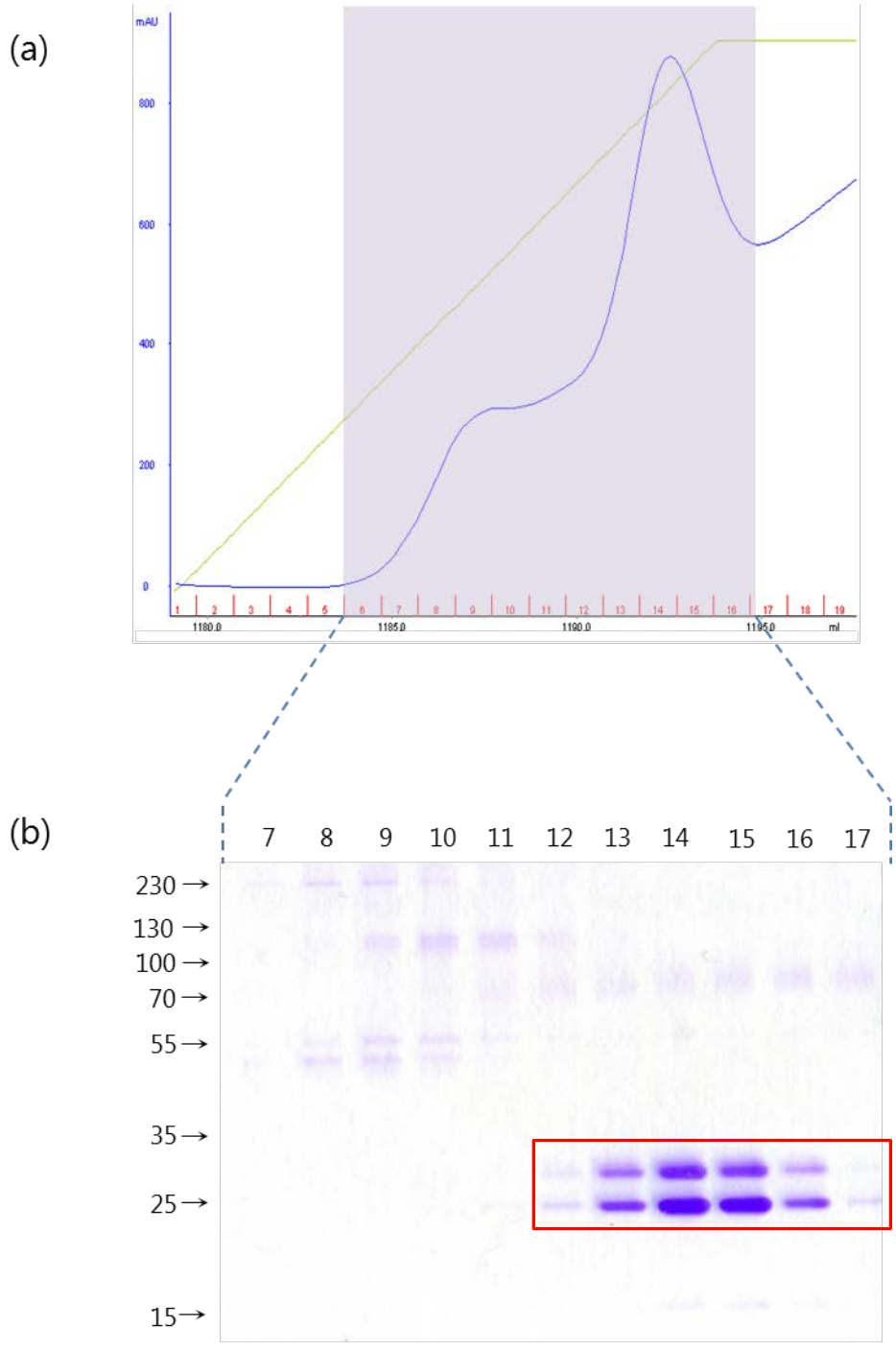


Figure 4.17: HisTag Purification of rCFHR2

(a) CHO/rCFHR2 tissue culture SN ran over a nickel-coated 5ml HiTrap™ Chelating columns, followed by an imidazole gradient elution (20-500mM). Columns were eluted in conjunction with the ÄKTA prime purification system (GE Healthcare). **(b)** Elution fractions 7-17 were electrophoresed by SDS-PAGE (10% gel), with subsequent staining with Coomassie blue. The red box indicates the elution fractions containing rCFHR2. These were collected, pooled and buffer exchanged into PBS/NaN_3 0.01%.

4.4.11 Quantification of CFHR4 in human sera

CFHR4 mAb R4/244 was selected to screen a preliminary population of aHUS and BTS control plasma. The primary aim was to determine whether a sandwich ELISA could detect CFHR4 in human plasma, and ascertain what variation exists within, and between, aHUS and normal controls. After setting up a CFHR4B standard curve, serum samples were loaded into the sandwich ELISA at a 1/20 dilution. The results show consistent levels of CFHR4 in the BTS and aHUS populations (**Figure 4.18**). The data from each group were compared using a Mann-Whitney test, as previously described (**Section 2.6.3**). The results indicate that the change between the 2 groups is not significant (p = >0.05%)

The aHUS population contained 3 serum samples heterozygous for the novel CFHR1/4 deletion. It was hypothesised these patients would have 50% less CFHR4 than the other patients who all have 2 copies of CFHR4. However, no significant decrease in CFHR4 levels was observed in these patients.

Using CFHR4B in the standard curve resulted mean concentration values of ~40 μg/ml in both the BTS and aHUS populations.

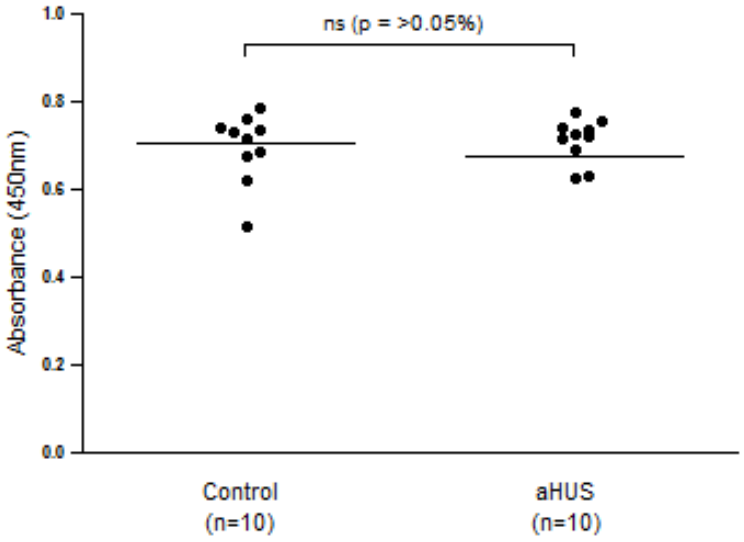


Figure 4.18: Detection of CFHR4 in human plasma

A group of aHUS and BTS plasma controls were screened to detect CFHR4. Plates were coated with R4/244 at 5 µg/ml. Plates were then blocked with 1% BSA/PBS, followed by detection using a CFHR4 rabbit pAb. Final detection was achieved using an HRPO-conjugated goat anti-rabbit Ig. A Mann-Whitney test was used to determine whether there was a significant change between the 2 groups. Horizontal line refers to median value in each population.

Key: ns; not significant.

4.5 Discussion

The aim of this chapter was to produce a set of mAbs against rCFHR1, rCFHR3 and rCFHR4B, and utilise their specificity to develop CFHR-specific detection assays using ELISA.

The production of an anti-CFHR1 mAb (R1/1037) was successful. However, cross-reactivity testing revealed that R1/1037 cross-reacts with native CFHR2. As R1/1037 does not cross-react with CFH, this suggests the binding epitope lies within CFHR1 SCR domains 1-2. Due to high degree of homology between SCR domains 1-2 of CFHR1 and CFHR2 (~99%), it is difficult to identify the particular location, or binding epitope of R1/1037.

Unfortunately, the dual binding capacity of R1/1037 means it is not suitable to specifically measure rCFHR1 protein concentration in human serum. To be used as a capture Ab, CFHR2 is also going to competitively bind and influence the signal result when using either a CFH or CFHR1 polyclonal. It could be argued that the ratio of CFHR1/CFHR2 binding to the R1/1037 coat is going to be maintained at a certain ratio. However, as we do not know the exact concentration of CFHR1 or CFHR2 in human serum, this ratio is difficult to determine. However, as I have produced both these proteins recombinantly there is the possibility of experimenting with different ratios in the standard curve.

It appears the only way to build a truly specific CFHR1 ELISA is by targeting the Ab response directly to the single amino acid changes that occur between CFHR1 and CFHR2 (SCR domain 1), and CFHR1 and CFH (SCR domains 4-5 and 19-20, respectively). My early work on producing CFHR1-specific mAbs did focus on the latter two fragments. However, after a failed attempt it was deemed more suitable to use full-length rCFHR1 to immunise, as this would result in a larger repertoire of mAbs, binding to a selection of epitopes.

I would conclude that immunisations using peptide fragments would be the most suitable approach to gain highly-specific mAbs against CFHR1.

The production of mAbs against CFHR4B was also successful. After specificity testing, it became clear that R4/244 was highly-specific for CFHR4, both the recombinant (rCFHRB) and native CFHR4A.

However, western blot data yielded an unusual result, whereby only CFHR4A was detected, but not CFHR4B. In human serum there are two isoforms of CFHR4 due to alternative splicing of the *CFHR4* RNA gene transcript. The resulting transcripts are referred to as *CFHR4A* and *CFHR4B*. Therefore, once translated CFHR4A consists of a duplication of SCR domains 1-4, at SCR domains 5-8, with the addition of SCR domain 9[165,166] (**Figure 1.3**). CFHR4B is identical to CFHR4A SCR domains 5-9. Based upon this sequencing data, CFHR4B was selected as the most suitable CFHR4 isotype for the aims of this work. Sequencing data suggests CFHR4B will contain the same antigenic peptides as CFHR4A, for use as an antigen for raising an Ab response and in detection of autoantibodies in aHUS patients (**Chapter 5**). It is currently unknown whether the duplication of SCR domains 1-4 in CFHR4A are involved in creating unique epitopes, relative to CFHR4B. Based upon structure/function models, it is thought that CFH does not have a tertiary protein structure, but rather the extended chain of SCR domains arrange themselves to recruit and secure CFH to the complement activated (C3b deposition) host cell surface[125]. There is currently no evidence to suggest the CFHR proteins have a tertiary structure that would result in a discontinuous antigen epitope from the interaction of different SCR domains (again, relative to CFHR4B). Additionally, it is well documented that the lower the molecular weight of a protein, the higher the chance of successful production in a recombinant system[215-217]. CFHR4B is essentially the latter part of CFHR4A, so an Ab response to CFHR4B should also result in cross-reactivity with CFHR4A. However, using CFHR4B allows the extra potential to produce a mAb to target CFHR4B, but not CFHR4A. Therefore, if the western blot result had detected CFHR4B, but not CFHR4A, this would fit with CFHR4B SCR domain 5-specificity.

Aside from antigenicity, it may be due to the availability of CFHR4B on the western blot, due to CFHR4B existing at a lower concentration (undetectable *via* western blot) in human serum, or the splice-variant may only be produced under certain conditions (e.g. under specific immunological signalling). Recent

evidence suggest CFHR4 plays a role in promoting complement activation[168], rather than regulating like its family members CFH, CFHR1 and CFHR5. There may be a role of CFHR4B that is only required when complement is activated. It remains unclear why CFHR4A would be detected, over its immunogen counterpart. However, the other CFHR4B mAbs, also cross-react with CFHR4B. It is clear that between R4/277 and R4/123, there is large difference between the band intensity between the two blots. This suggests these mAbs may bind CFHR4B with a different level of intensity. This is some evidence to suggest CFHR4B exists as a homodimer[166], which would explain the larger band. The difference between CFHR4A and a potential CFHR4B dimer is one SCR domain; the dimer will contain an additional SCR 9. This only confers a 6-7 kDa change in molecular weight so is not going to resolve on a 10% SDS-PAGE gel. However, reducing, boiling and treating serum with SDS failed to breakdown potential CFHR4B homodimers.

R1/1037 was essentially deemed unsuitable for use in a sandwich ELISA for determination of CFHR1 concentration, due to cross-reactivity with CFHR2 (as previously described). However, the reagent was fortuitously useful in the detection of rCFHR2, resulting in the progression of this work. We now have a full panel of CFHR proteins available for autoantibody screening of aHUS and IgAN patient sera. The Ab is also a useful reagent to have in purifying CFHR1 from human serum. Clearly, CFHR2 is also purified, but a second round of purification *via* a high resolution gel filtration column would separate out these proteins. Alternatively, the Ab could be used to purify CFHR2 from CFHR1 deficient serum (*CFHR1-/-* aHUS patients). Additionally, R1/1037 Ab may prove useful for creating CFHR1/2 deficient serum, allowing experimental focus to be placed upon other CFH family proteins. R4/244 could also be used in this way, and in conjunction with R1/1037.

The mAbs produced can also be utilised in the identification and purification of CFH family fusion proteins. NAHR is commonplace within the RCA gene cluster, resulting in copy number variation and fusion events in *CFH* family genes. One of the difficulties with assessing the functionality of gene fusions is the difficulty in purifying them from patient serum. R1/1037 is another potential mAb that can be used to study the effect of fusion proteins, enabling specific

purification of CFH/CFHR1 fusions with a suitable CFHR1 binding epitope (without cross-purification of CFH). Any CFHR2 cross-reactivity can again be addressed using high resolution gel filtration.

The specificity of the R4/244 mAb was used in an optimised sandwich ELISA to detect plasma CFHR4. R4/244 was used as the coating/capture Ab, and rabbit anti-CFHR4 polysera (produced in-house) used to for detection. Based upon the specificity testing data, this Ab is only detecting CFHR4A, but not native plasma CFHR4B. However, R4/244 does detect the rCFHR4B antigen to which it was raised. It remains unclear why native CFHR4B is not detectable, as R4/277 and R4/123 appear to detect it successfully. Potentially, the single point mutation in the rCFH4B is recognised by R4/244 which would influence its affinity in binding to native CFHR4B. This effect may be offset by the tertiary structure of native CFHR4A, allowing a higher affinity interaction. Domain swaps and epitope mapping could be used to address this theory.

It is important to recognise that using rCFHR4B to create a standard curve for this assay may not yield the most accurate results for measuring CFHR4 concentration. The primary reason being that CFHR4 exists as CFHR4A and CFHRB, and potentially as a CFHR4B homodimer (as previously described). Therefore, when measuring CFHR4 concentration it is difficult to ascertain how these variables will compare to a standard curve using just one CFHR4 species. However, using this assay to compare a patient and a normal control group is still a viable assay as the data from each group is relative. Taking these confounding variables into consideration, the R4/244 sandwich ELISA was able to detect CFHR4 at levels consistent with a previously reported CFHR4 concentration (~40 µg/ml)[168].

The remaining CFHR4 mAbs, R4/277 and R4/123 are yet to be testing as capture Abs. Based upon the specificity testing results, R4/277 would also serve as a suitable candidate capture Ab in this type of assay. However, R4/123 cross-reacts with CFH so could not be used in conjunction with the rabbit anti-CFHR4 polysera (also binds CFH).

In summary, several mAbs have been produced which can be readily utilised in a variety of experiment procedures within this research laboratory, including

ELISA and western blot analysis of tissue culture, purified, and plasma proteins. Based upon the research aims of this chapter, future work will involve further optimisation of the CFHR1 detection ELISA (using R1/1037), and screening of larger cohorts of disease and control plasma.

Chapter 5

5. CFH family autoantibodies in aHUS and IgAN

5.1 Introduction

CFH autoantibodies have been described in approximately 10% of aHUS patients[162,178,218,219]. Based upon the high level of amino acid sequence homology between CFH and the CFHR proteins, the CFHRs are therefore also candidates for an autoantibody response; either as the primary trigger for the Ab response, or as secondary to CFH autoantibody cross-reactivity. There is evidence to suggest CFH autoantibodies are functionally significant in aHUS disease pathogenesis, i.e. the Ab binding is predominantly to the C-terminus of CFH where aHUS associated mutations are known to cluster[162,220]. Additionally, CFH autoantibodies have been shown to disrupt CFH-mediated complement regulation in AP haemolytic assays[221]. The degree to which autoantibodies are involved in disease outcome is currently unclear. However, based upon the evidence presented to-date, CFH autoantibodies are likely to be a contributing factor in the pathogenesis of a subset of aHUS patients[179].

Autoantibodies targeting CFHR1 have been identified, but only in the DEAP-HUS (**Section 1.6.6**) sub-population of patients[179,222]. Autoantibodies against the remaining CFHR proteins are yet to be found. CFH autoantibody positive plasma has been screened for cross-reactivity to the CFHR proteins, but with no further evidence of cross-reactivity with CFHR proteins 2-5. However, there is a currently no data presented whereby a full aHUS cohort (CFH autoantibody positive and negative patients) has been screened against each CFHR protein.

In IgAN patients, IgA Abs are found deposited on the mesangial surface of kidney glomeruli. However, the antigen-specificity of these IgA Abs is currently unknown. The IgA found deposited is polymeric IgA1 (pIgA1). It is well documented that pIgA1 in IgAN patients is abnormally glycosylated at the hinge region of the Ab heavy chain. This glycosylation results in increased affinity for the mesangium, hence deposition.

Complement proteins (activatory and regulatory) are found co-localised with deposited pIgA1, suggesting IgA deposition may be resulting in complement

activation. IgA is a poor activator of complement so could not be solely responsible for triggering activating complement. However, IgG (and IgA) autoantibodies against pIgA1 were also found in the immune complexes, suggesting IgG as the activator of complement *via* the CP.

The pIgA1 complex may also be deposited on the mesangium due to antigen binding, related to an autoimmune response to mesangial-associated antigens. Therefore, screening for autoantibody-specificity is an important step in understanding the complexity of IgAN.

There may be a role for the CFHR proteins in the pathogenesis of IgAN. CFHR5 has been found deposited in the glomerulus of IgAN patients, suggesting a role for the protein in protecting or damaging the glomerulus. CFHR5 has been shown to exhibit both cofactor and decay-accelerating activity, so the role in IgAN is more likely to be protective (regulating complement activation). However, regulation does not appear to be efficient enough to stop the progression of disease. In CFHR5 nephropathy, a duplication of CFHR5 SCR domains 1-2 appears to be the only triggering factor that correlates with disease. This suggests CFHR5 may play a role in protecting the kidney from complement-mediated attack.

The detection of IgA and IgG deposited on the mesangial surface may not be restricted to the autoimmune response to abnormally glycosylated pIgA1. There may also be autoantibodies targeting other mesangial surface antigens, namely complement regulatory proteins.

There is increasing evidence of autoantibody responses against complement regulators in renal disease. Therefore, it is reasonable to hypothesise this about IgAN, a disease where there is evidence of autoantibodies and altered complement regulator expression in the diseased kidney[82-84,105].

As CFH has shown significant antigenicity in ‘autoimmune’ aHUS, CFH and the CFHR proteins are suitable candidates for IgA autoantibody screening. The pIgA1 Ab is also found in the circulation, so blood screening is a suitable place to begin.

5.2 Chapter Aims

To develop ELISA protocols for screening human plasma for autoantibodies against CFH family proteins allowing aHUS and IgAN patient cohorts to be screened for the presence of autoantibodies to CFHR1, CFHR2, CFHR3, CFHR4B and CFHR5.

5.3 Results

5.3.1 CFH fragment screening: CFH autoantibody binding specificity

The presence of CFH autoantibodies were first identified in a French aHUS patient cohort[162] However, it was unclear whether these autoantibodies were involved in the pathogenesis of disease, or were merely an epiphenomenon seen in ~10% of aHUS patients. To begin to understand the potential role that CFH autoantibodies play in aHUS pathogenesis, truncated CFH fragments were used to assess which region of CFH was being targeted. Previously defined CFH autoantibody positive patients from our Newcastle aHUS cohort were used in this analysis[162]. CFH fragments consisting of SCR domains 1-4, 8-15 and 19-20 (kind gift from Prof. Paul Barlow, Edinburgh University) were coated in equimolar concentrations to an ELISA plate, followed by washing and blocking as previously described (**Section 2.3.4.1**). CFH autoantibody positive plasma samples were applied in-triplicate at a 1/50 dilution, followed by detection with HRPO-conjugated sheep anti-mouse IgG.

From the 12 patients tested, 6 have a CFH autoantibody response which targets the CFH_{19-20} fragment (P2, P4, P7, P8, P10, and P11), 1 patient had specificity for CFH_{1-4} (P6), 1 patient for CFH_{8-15} (P12), and 3 patients with no specificity for the CFH fragments presented (**Figure 5.1**). These results indicated that in at least 50% of CFH autoantibody positive aHUS patients, the Ab is targeting the C-terminal SCR domains 19-20. This is main region involved in CFH binding to heparin, or the cell surface[223,224].

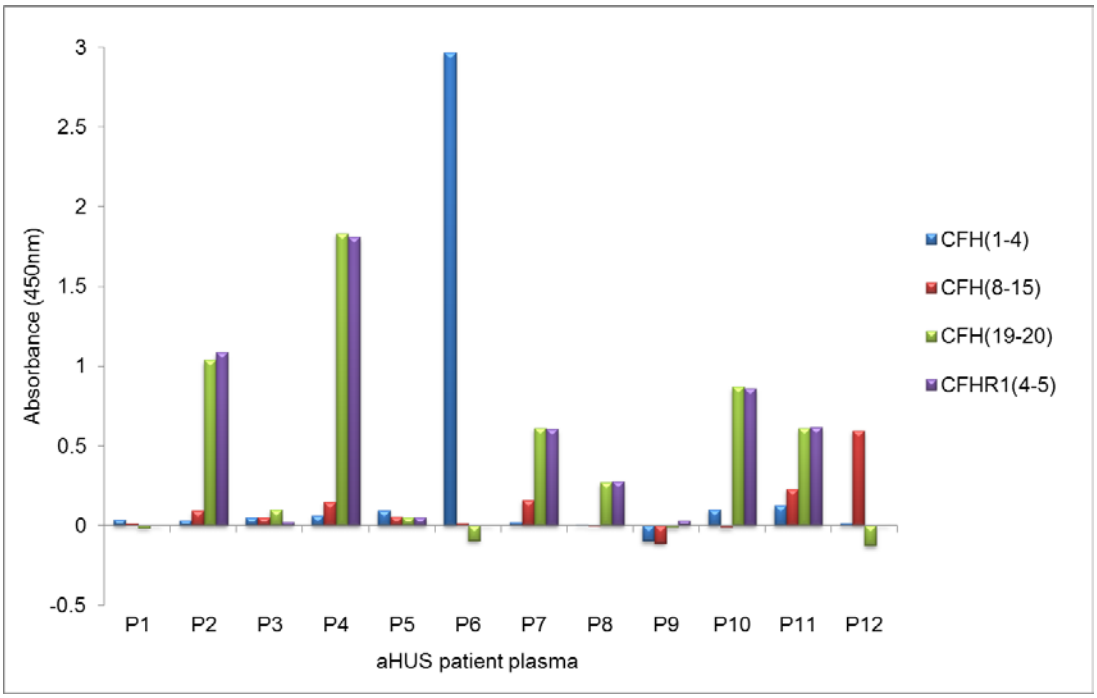


Figure 5.1: CFH autoantibody binding to CFH fragments

ELISA plates were coated with equimolar concentrations of CFH fragments, CFH_{1-4}, CFH_{8-15}, CFH_{19-20} and CFH_{4-5}. Plates were blocking with 1% BSA/PBS, followed by the addition of a 1/50 dilution of patient sera. CFH IgG autoantibodies were detected using an HRPO-conjugated goat anti human IgG secondary Ab (1/4,000).

5.3.2 CFH autoantibody detection ELISA optimisation

A full panel of rCFHR proteins were produced (**Chapter 3**) to screen human plasma for the presence of autoantibodies to CFH family proteins. As previously observed in our group, using human plasma in ELISA requires careful optimisation. For each rCFHR protein, a unique ELISA optimisation was completed to select the most suitable conditions for each screen (**Section 2.5.2**). Data from the optimisation of the rCFHR1 ELISA screen has been selected as a typical representation of the optimisation process (**Figure 5.2-3**).

5.3.2.1 CFHR1 autoantibody ELISA optimisation (Stage 1)

A multiplex-ELISA was designed to assess the binding efficiency of CFH to 3 different ELISA plate surfaces, using 3 different coating buffers and a range of rCFHR1 coat concentrations, as previously described (**Section 2.5.2**). Bound rCFHR1 was detected using an antigen-specific primary Ab. Binding of the primary Ab to the coated antigen (i.e. rCFHR1) may be altered depending on the biophysical interaction of the antigen with the plate surface. Therefore, this preliminary optimisation step is a guide to choosing the most appropriate coating buffer and coat concentration. Any potential autoantibodies are not necessarily going to bind to the same epitope as this primary Ab.

The results indicate the highest signal, with the lowest concentration of coated rCFHR1 comes from using the ‘flexiplate’ in-conjunction with PBS buffer (**Figure 5.2**). The rCFHR1 signal was saturated at 1 µg/ml (A_{280} = 2.253), which is ~17% higher than the nearest rival plate/coating buffer combination at 1 µg/ml. These rCFHR1 coating conditions were used in ‘Stage 2’ of the optimisation procedure.

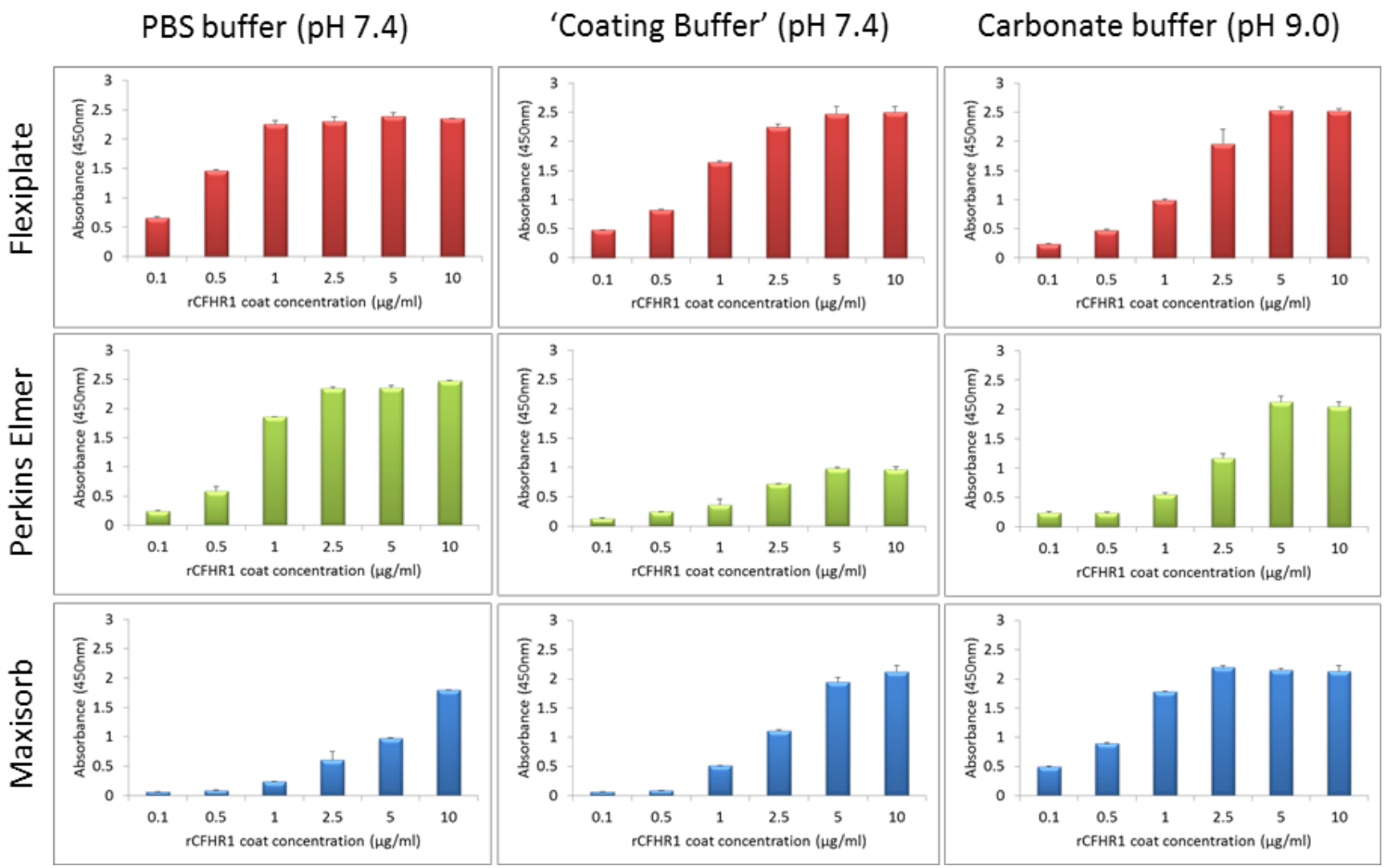


Figure 5.2: Stage 1 of rCFHR1 autoantibody ELISA optimisation

Stage 1 assessed rCFHR1 coating to 3 different plate surfaces (Flexiplate, Perkins Elmer and Maxisorb), with 3 different antigen coating buffers (PBS, 'Coating Buffer', and carbonate buffer), using a range of rCFHR1 coating concentrations (0.1-10 µg/ml). Plates were coated overnight with rCFHR1, then blocked with BSA blocking buffer (PBS, 1% BSA, pH 7.4). Coating efficiency was assessed using C18/3 (1/2,000 dilution), followed by detection with HRPO-conjugated sheep anti-mouse IgG. The highest absorbance reading prior to signal saturation was considered the most appropriate conditions to carry forward to 'Stage 2' testing.

5.3.2.2 CFHR1 autoantibody ELISA optimisation (Stage 2)

Optimal conditions for coating rCFHR1 to the ELISA plate surface were established above. However, optimisation was required to assess how the plate blocking and human plasma would interact in these assays and determine the level of background binding seen. Therefore, the same 3 plates surfaces were assessed, in-conjunction with 3 different blocking reagents, and the addition of human plasma diluted 1/50 (essentially, serving as primary Ab). Based on the study by Moore *et al*. (2010)[162], 99% of Blood Transfusion Service (BTS) plasma collected from healthy blood donor controls are found negative for CFH autoantibodies. Therefore, 10 test samples (BTS1-10) were selected to identify conditions whereby the proposed negatives give low, or no response in the assay. An IgG CFH autoantibody positive plasma sample was used as a positive control for binding to rCFHR1.

After calculating the mean value of each BTS population in each set of conditions, the mean value closest to zero (A_{280}) came from conditions using the 'flexiplate' surface in-conjunction with 1% BSA blocking buffer (**Figure 5.3**). As a secondary measure for condition selection, the CFH autoantibody positive control values across all conditions were assessed. The highest signal came from the 'flexiplate' surface, using 1% BSA blocking buffer (as before).

Absorbance (450nm)
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
BTS1
BTS2
BTS3
BTS4
BTS5
BTS6
BTS7
BTS8
BTS9
CFH+
Control 1
Control 2
Control 3

Figure 5.3: Stage 2 of rCFHR1 autoantibody ELISA optimisation

'Stage 2' assessed 3 different plate surfaces (Flexiplate, Perkins Elmer and Maxisorb), with 3 different blocking buffers, (1% BSA, 1/5 dilution of 'Ultrablock', and 0.1% Tween20), using plasma from 10 BTS controls dilution 1/50 with PBS (serving as 'primary Ab'). Bound IgG was detected using HRPO-conjugated goat anti-human IgG. 'Stage 2' testing included non-coated duplicate plates (to assess background signal) which was then subtracted from rCFHR1 coated plate data. The data presented is background subtracted. Controls: CFH autoantibody positive plasma (CFH+), C18/3 positive coat control (Control 1), secondary only HRPO-conjugated goat anti-human IgG (Control 2), and secondary only HRPO-conjugated sheep anti-mouse IgG (Control 3). The BTS population mean closest to zero (A_{280}), and the highest 'CFH+' positive control value were considered when selecting the most appropriate conditions to use for autoantibody ELISA screening.

5.3.3 Screening for autoantibodies against CFH family proteins

The presence of anti-CFH autoantibodies (IgG detection) had previously been assessed in our laboratory, and the results were subsequently published[162]. This, and previous studies, suggested a level of cross-reactivity with CFHR1 and therefore, aHUS IgG autoantibody screening was carried out against each of the CFHR proteins.

Autoantibodies detection in IgAN patient plasma is more focused upon IgA detection, on account of IgA (pIgA1) deposition in kidney glomeruli, and also in fluid-phase circulation. The antigenicity of pIgA1 is currently unknown, but based upon the co-localisation of both C3 and CFHR5 in the mesangium of IgAN patients suggests an involvement of complement proteins. The similarities between aHUS and IgAN suggest the possibility that autoantibodies to CFH or CFHR5 could arise or be associated with IgAN. In order to test this hypothesis, patients from both cohorts were screened.

5.3.3.1 Screening for CFHR1 autoantibodies in aHUS and IgAN

The terminal SCR domains of CFH and CFHR1 share a high degree of sequence homology (as previously described), so the previously observed CFH autoantibody response against CFH SCR domains 19-20 and CFHR1 domains 4-5 (Figure 5.1) suggested full length rCFHR1 may also yield a positive result in these patients.

The ELISA screen was completed using the methodology previously described (**Section 2.3.4.1**), using the predetermined parameters previously outlined. The 74 patients in this screen were aHUS patients with a confirmed diagnosis, from Newcastle aHUS cohort[162]. To determine a threshold between a positive and negative result, 100 blood donor controls were screened and the A_{450} population mean value, plus 2 standard deviations determined. Any absorbance reading above this threshold was considered positive, and any below was considered negative. Based upon these criteria, the aHUS cohort yielded 3 positive patient samples (**Figure 5.4a**). These patients had been previously been identified as CFH autoantibody positive, with specificity for $CFH_{19\text{-}20}$ and $CFHR1_{4\text{-}5}$. Within this cohort of 74 aHUS patients, there was one other CFH autoantibody positive patient sample expected to be CFHR1 autoantibody

positive. However, analysis of patient plasma during this screen yielded a negative result. This patient was positive for full-length CFH, and positive for the $rCFH_{19-20}$, and $rCFHR1_{4-5}$ fragments, so it unclear why the plasma sample is negative for full-length rCFHR1.

An IgAN patient cohort of 22 patients was randomly selected and screened against rCFHR1, testing for the presence of either IgG (**Figure 5.4b**) and IgA autoantibodies (**Figure 5.4c**). As previously described, a panel of 100 BTS controls were screened to determine a threshold for positivity in the ELISA. The results show no autoantibodies, of either IgG or IgA subclass, could be detected against rCFHR1 using this ELISA screen.

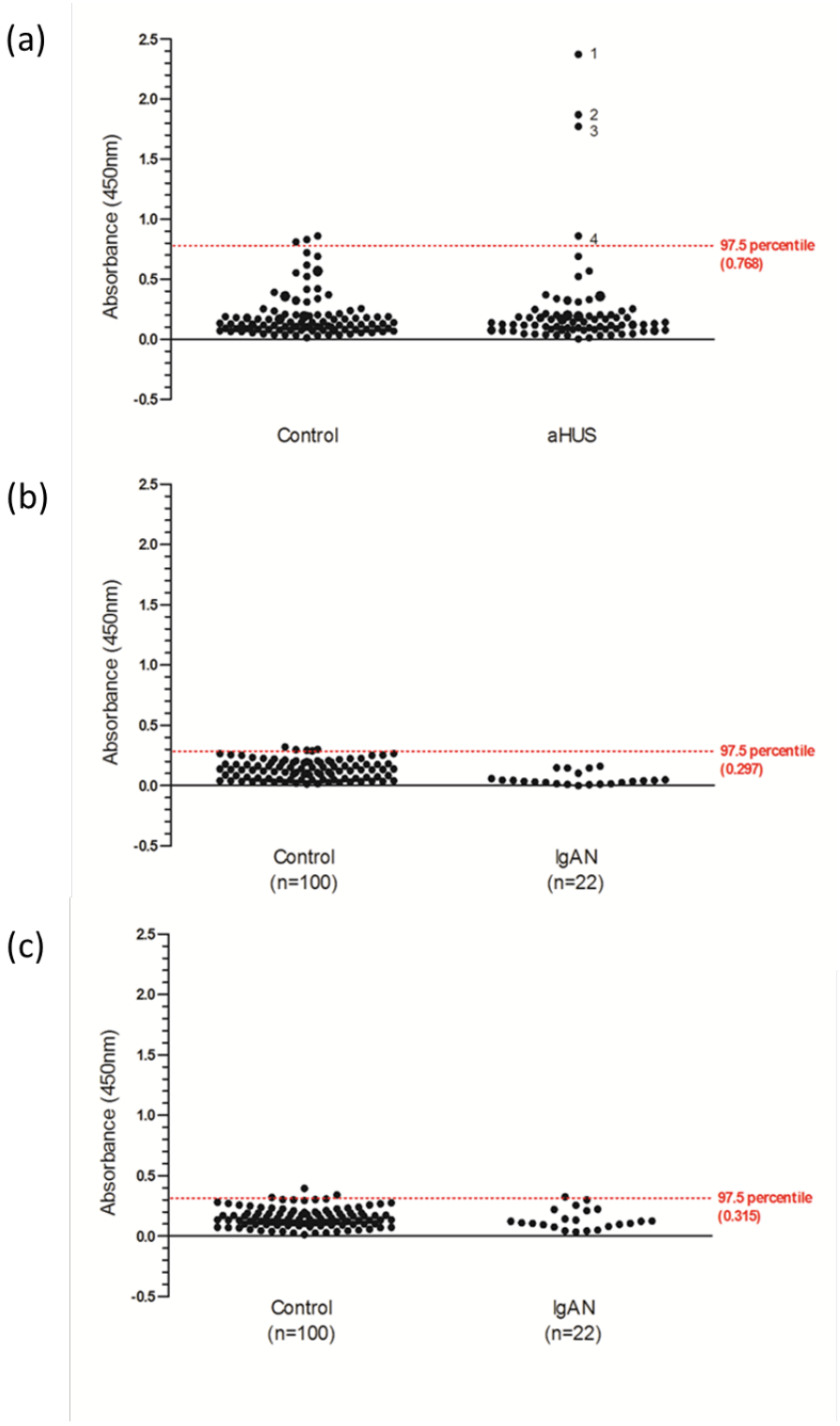


Figure 5.4: rCFHR1 autoantibody screen in aHUS and IgAN patients

rCFHR1 was coated to ELISA flexiplates, followed by blocking with 1% BSA blocking buffer. Patient and control plasma were diluted 1/50 and applied in-duplicate to the coated and non-coated plates. No coat plate absorbance readings were subtracted from rCFHR1 coat readings and plotted above. **(a)** CFHR1 IgG autoantibodies in aHUS and BTS. **(b)** IgAN and BTS control plasma cohorts were screened for CFHR1 IgG autoantibodies. **(c)** IgAN and BTS control plasma cohorts were screened for CFHR1 IgA autoantibodies.

5.3.3.2 CFHR1 autoantibody screen in aHUS patient plasma

As noted above, the aHUS screen for anti-CFHR1 autoantibodies yielded 3 positive results (**Figure 5.4a**). To support previous data of rCFHR1 autoantibody positivity, patient plasma was tested *via* western blot. The rCFHR1 protein was loaded onto a 10% SDS-PAGE gel and transferred to nitrocellulose, followed by blocking with NFDM blocking buffer. The nitrocellulose was cut into strips and each strip incubated with the ELISA positive patient plasma, as previously described (**Section 2.3.6**). The strips were then washed and probed for the presence of bound human IgG.

All 3 positive patients from the ELISA screen were also found to be positive in the western blot screen (**Figure 5.5**). This provides further validation of an anti-CFH/rCFHR1 autoantibody response in these patients. It also validates the ability of the rCFHR1 autoantibody ELISA protocol to detect positive patients. It is important to note, each of these positive patients had the characteristic *CFHR3/1* gene deletion[162]. Therefore, this is an anti-CFH autoantibody response, not an anti-CFHR1 response. Cross-reactivity is merely coincidental based up the sequence homology between the two proteins. No aHUS patients were found with solely with an anti-CFHR1 autoantibody positive response.

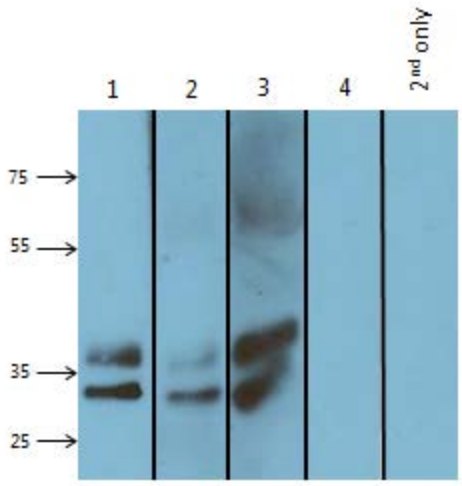


Figure 5.5: Western blot screen of rCFHR1 autoantibodies in aHUS

rCFHR1 autoantibody positive plasma samples from the ELISA screen were re-screened *via* Western blot. rCFHR1 was separated on a 10% SDS-PAGE gel, transferred to nitrocellulose and lanes were cut into individual strips. Each strip was individually incubated with each rCFHR1 autoantibody positive plasma (diluted 1/50), followed by detection with HRPO-conjugated goat anti-human IgG (1/10,000 dilution). aHUS patients are numbered 1-4, as before (Figure 5.4a) The strip blots shown are from film exposed for 30 secs.

5.3.3.3 CFHR2-4B autoantibody screens in aHUS and IgAN plasma

Autoantibody ELISA screens were completed against rCFHR2, rCFHR3 and rCFHR4B using the protocol and parameters previously described (**Section 2.5.2**). The results for IgG and IgA autoantibody detection in both patient cohorts yielded negative results for CFHR2 (**Figure 5.6**), CFHR3 (**Figure 5.7**) and CFHR4B (**Figure 5.8**).

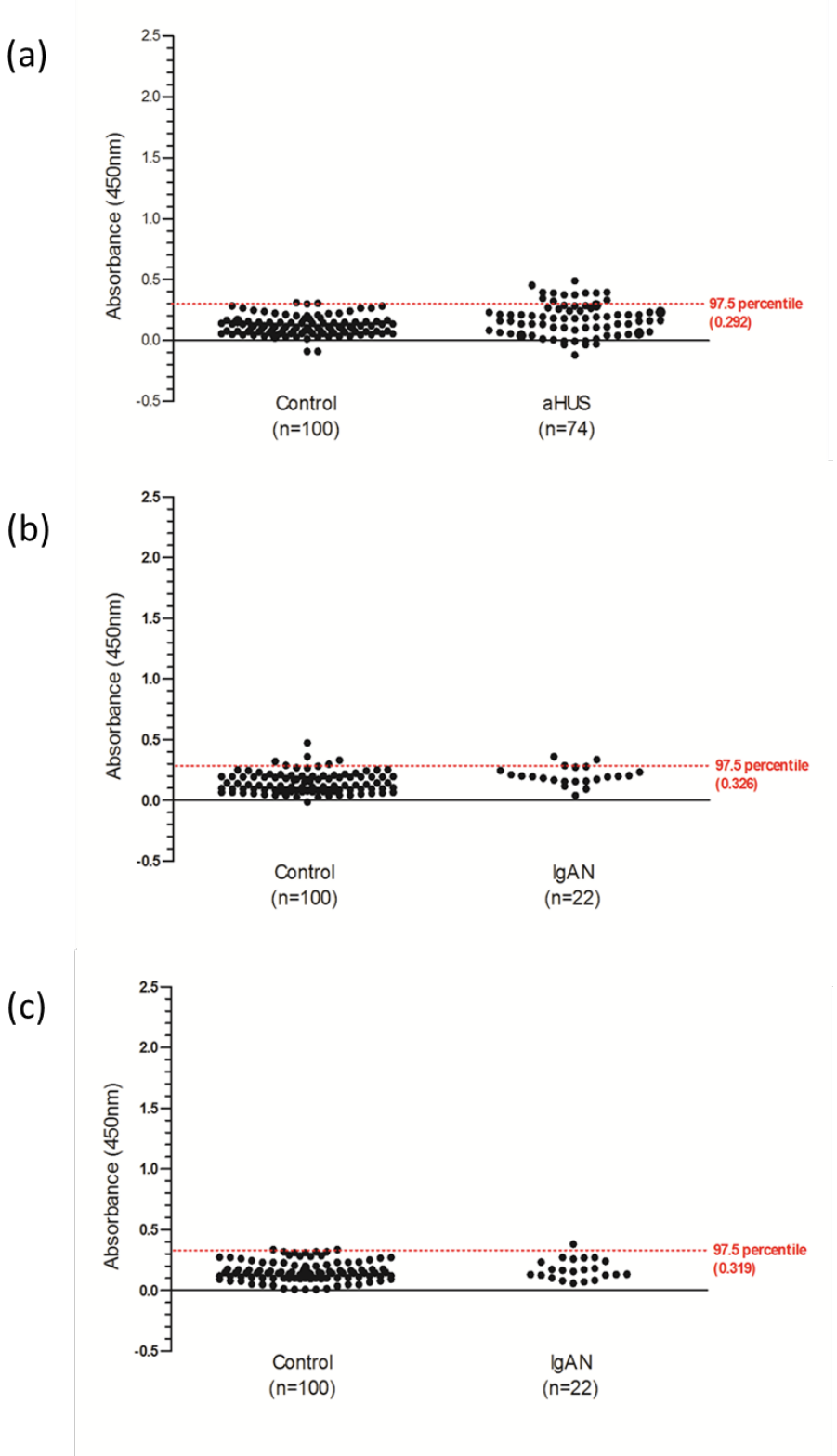


Figure 5.6: rCFHR2 autoantibody screen in aHUS and IgAN patients

rCFHR2 was coated to ELISA flexiplates, followed by blocking with 1% BSA blocking buffer. Patient and control plasma were diluted 1/50 and applied in-duplicate to the coated and non-coated plates. No coat plate absorbance readings were subtracted from rCFHR2 coat readings and plotted above. **(a)** CFHR2 IgG autoantibodies in aHUS and BTS. **(b)** IgAN and BTS control plasma cohorts were screened for CFHR2 IgG autoantibodies. **(c)** IgAN and BTS control plasma cohorts were screened for CFHR2 IgA autoantibodies.

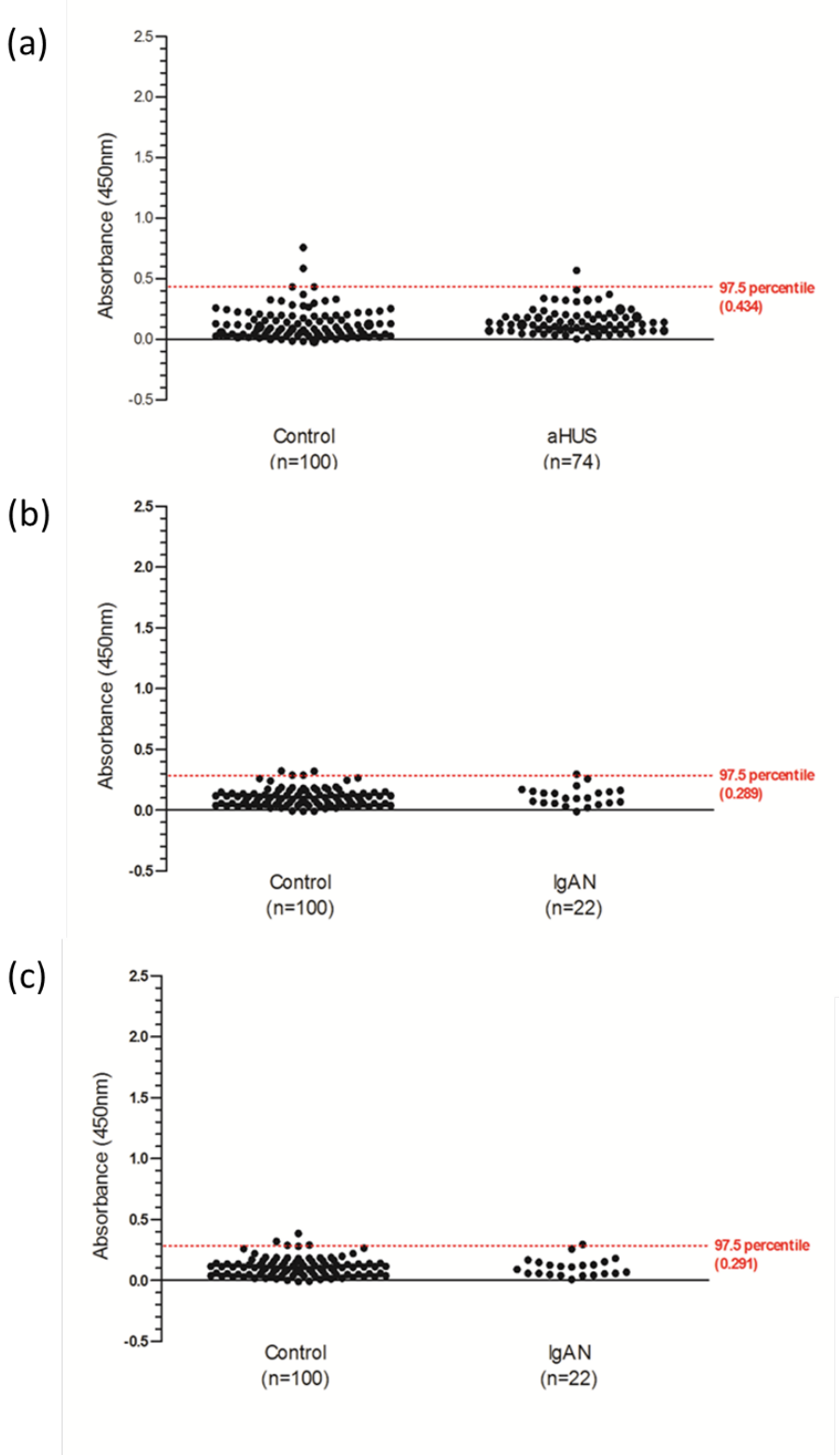


Figure 5.7: rCFHR3 autoantibody screen in aHUS and IgAN patients

rCFHR3 was coated to ELISA flexiplates, followed by blocking with 1% BSA blocking buffer. Patient and control plasma were diluted 1/50 and applied in-duplicate to the coated and non-coated plates. No coat plate absorbance readings were subtracted from rCFHR3 coat readings and plotted above. **(a)** CFHR3 IgG autoantibodies in aHUS and BTS. **(b)** IgAN and BTS control plasma cohorts were screened for CFHR3 IgG autoantibodies. **(c)** IgAN and BTS control plasma cohorts were screened for CFHR3 IgA autoantibodies.

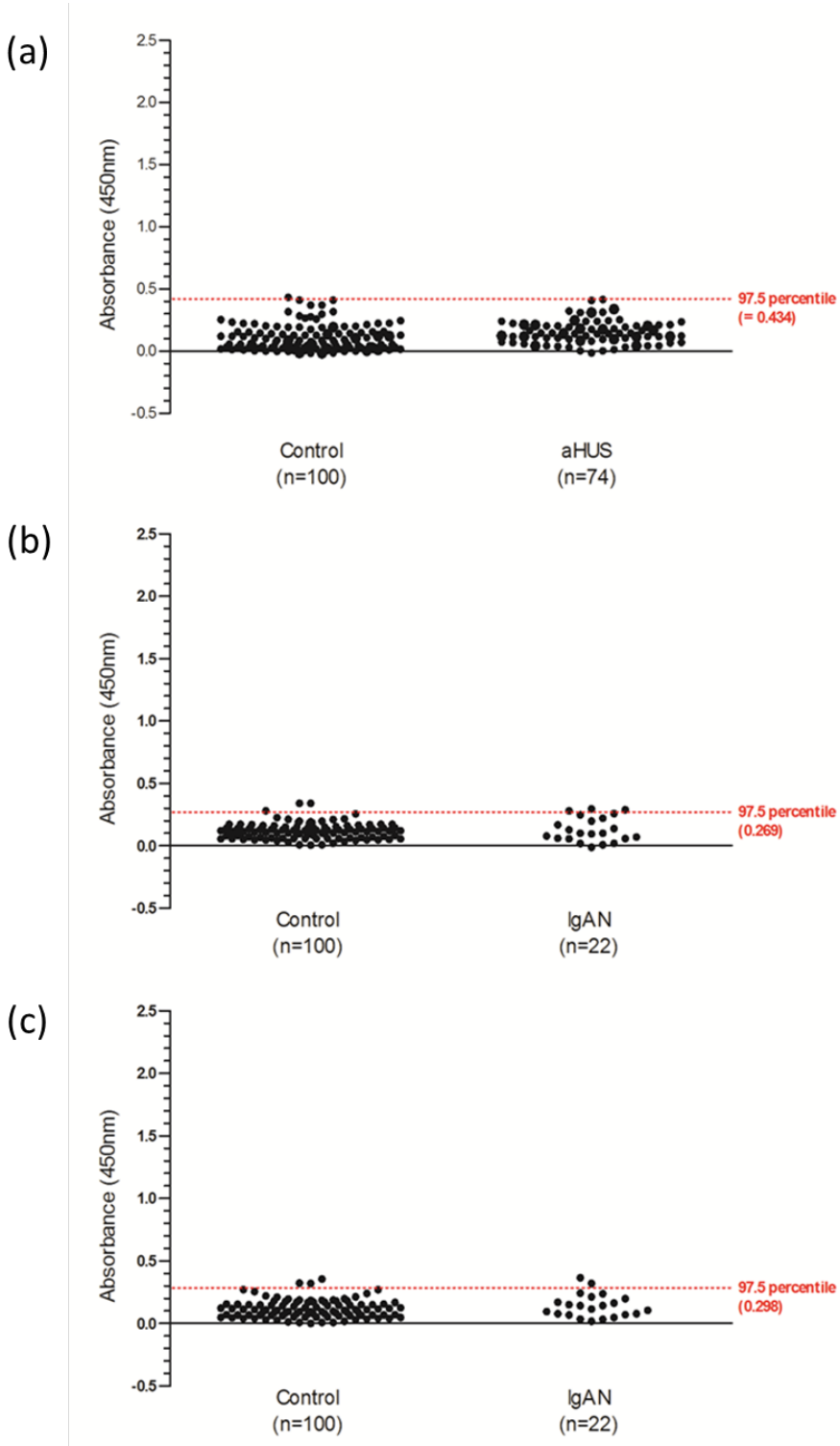


Figure 5.8: rCFHR4B autoantibody screen in aHUS and IgAN patients

rCFHR4B was coated to ELISA flexiplates, followed by blocking with 1% BSA blocking buffer. Patient and control plasma were diluted 1/50 and applied in-duplicate to the coated and non-coated plates. No coat plate absorbance readings were subtracted from rCFHR4B coat readings and plotted above. **(a)** CFHR4 IgG autoantibodies in aHUS and BTS. **(b)** IgAN and BTS control plasma cohorts were screened for CFHR4 IgG autoantibodies. **(c)** IgAN and BTS control plasma cohorts were screened for CFHR4 IgA autoantibodies.

5.3.3.4 CFHR5 autoantibody screens in aHUS and IgAN patient plasma

No IgG subclass autoantibodies against CFHR5 could be detected when plasma samples from aHUS (**Figure 5.9a**), or IgAN (**Figure 5.9b**) patients were screened. However, in the IgAN patient screen, when detecting IgA subclass autoantibodies, 2 patient samples gave positive results (P5 and P14), equivalent to ~9.1% of this population (**Figure 5.9c**).

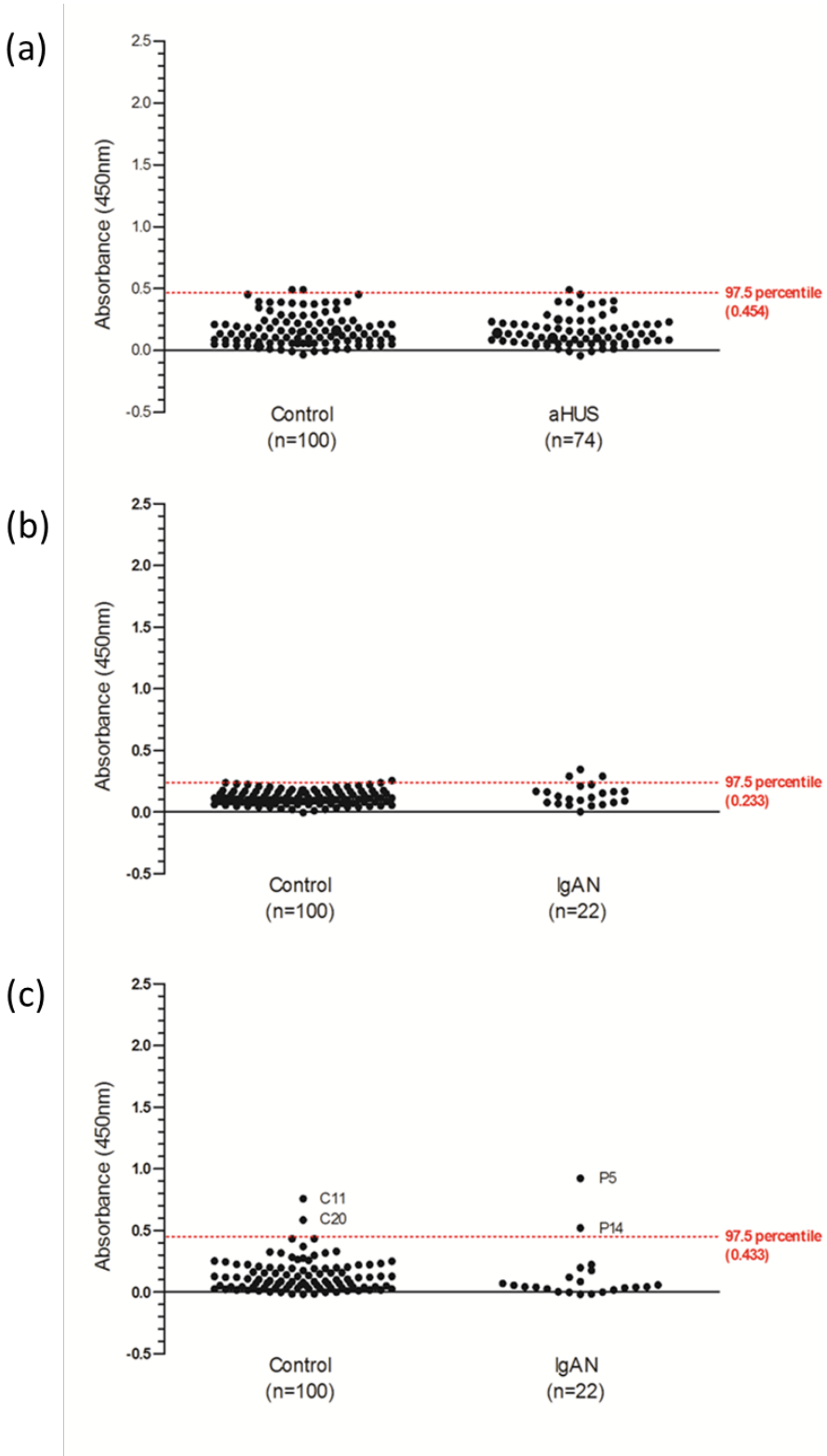


Figure 5.9: rCFHR5 autoantibody screen in aHUS and IgAN patients

rCFHR5 was coated to ELISA flexiplates, followed by blocking with 1% BSA blocking buffer. Patient and control plasma were diluted 1/50 and applied in-duplicate to the coated and non-coated plates. No coat plate absorbance readings were subtracted from rCFHR5 coat readings and plotted above. **(a)** CFHR5 IgG autoantibodies in aHUS and BTS. **(b)** IgAN and BTS control plasma cohorts were screened for CFHR5 IgG autoantibodies. **(c)** IgAN and BTS control plasma cohorts were screened for CFHR5 IgA autoantibodies.

5.3.3.5 Western blot screening of CFHR5 autoantibodies in IgAN plasma

To confirm the presence of anti-CFHR5 in IgAN patients, plasma from the positive patients were tested for reactivity with rCFHR5 *via* western blot, as previously described (**Section 5.3.3.2**). As the IgAN cohort is made up of just 22 samples, it is was manageable to analyse all patient samples using this method and determine the relative advantages compared to ELISA.

Plasma from 14 IgAN patients (1-2, 4, 8-16, 19-20) displayed reactivity (showing visible bands) in the rCFHR5 western blot screen (**Figure 5.10**). However, the band size in these 14 samples is significantly larger than estimated molecular weight of rCFHR5, and suggests this interaction is not to rCFHR5 but to a contaminating protein in the preparation. Initially, these results were considered false-positives. However, P5 displayed IgA reactivity to 3 distinct bands on the blot, with the strongest signal from the 'false-positive' band (labelled 'y'). The other bands corresponded to rCFHR5 (labelled 'z'), and a potential homodimer isoform of rCFHR5 (labelled 'x'). Therefore, at least 1 out of 22 IgAN patients appeared to exhibit an IgA autoantibody response to rCFHR5 in both the Western blot analysis, and the previous ELISA testing.

The high level of false positives in this screen raised questions regarding the level and source of the contamination in the rCFHR5 sample. It is not unusual that a purified protein sample would contain trace amounts of contaminating proteins. As highlighted in Chapter 3, any potential for interference is reduced when the sample is diluted for use in the ELISA screen. Aside from this, interference should be nullified by the combination of the ELISA and confirmation *via* western blot. Therefore, it was deemed essential to determine the origin of these contaminating proteins and therefore we had the rCFHR5 sample analysed using peptide mass fingerprinting (PMF).

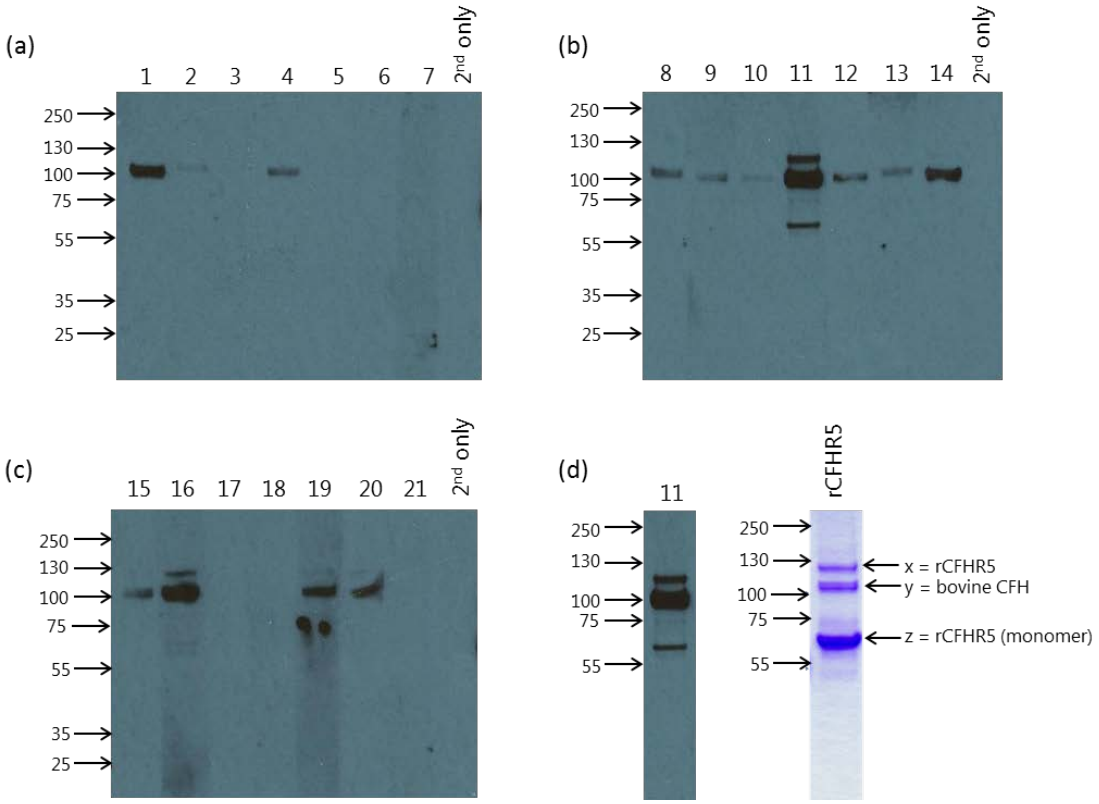


Figure 5.10: Strip blot of IgAN IgA autoantibodies against rCFHR5

(a-c) rCFHR5 was loaded on three 10% SDS-PAGE gels, transferred to nitrocellulose and lanes were cut into strips. Each strip was incubated with a 1/50 dilution of IgAN plasma (2ml volume), followed by detection with an HRPO-conjugated rabbit anti-human IgA (1/10,000 dilution). IgAN patients are numbered 1-21. A secondary only negative control was also included (2nd only). The strip blots shown are from film exposed for 10 min. **(d)** Patient 11 from panel **(b)** has been selected and compared to an SDS-PAGE separation of rCFHR5 (with Coomassie staining), after over-loading the protein to highlight contaminating protein bands. These bands were cut from the gel and identified *via* peptide mass fingerprinting (PMF). Band was named x, y and z prior to identification *via* PMF.

5.3.3.6 Identification of proteins in CFHR5 protein sample

To identify the origin of the contaminating bands in the purified rCFHR5 sample, the sample was 'overloaded' onto a 10% SDS-PAGE gel, separated by electrophoresis and Coomassie stained. This highlighted three bands (**Figure 5.10d**), each of which was individual cut from the gel and sent for PMF (Pinnacle, Newcastle University).

The PMF results identified band 'x' and 'z' as CFHR5, providing further confirmation of homodimeric and monomeric conformations of the protein, respectively. However, band 'y' was identified as bovine CFH. Bovine CFH will be a constituent of the CHO/rCFHR5 tissue culture SN, which is supplemented with 10% FCS. Bovine CFH is not routinely purified during a HisTag purification (at least at these levels) based upon other purifications of rCFHR proteins. This suggests that native bovine CFH (no HisTag) has complexed with human rCFHR5 and is subsequently, HisTag purified. The importance of this finding remains to be established.

Taking into consideration the homology between human and bovine CFH (~73%), the previous conclusion regarding the detection of 14 false-positive results (**Section 5.3.3.7**) is likely correct as 13 of the 14 patients do not have a CFHR5 autoantibody phenotype, but may interact with exogenous bovine CFH. Exposure to bovine CFH in milk or meat products could explain this reactivity. Again, the importance of such a finding is unclear. The anomalous false-positive results suggest screening IgAN plasma against human CFH and purified bovine CFH.

5.3.3.7 ELISA Screening for CFH autoantibodies in IgAN patient plasma

Optimisation of the ELISA screen for CFH was unable to identify suitable conditions for the ELISA screen of IgA autoantibodies. Further investigation is required to find suitable conditions for this assay.

5.3.3.8 Western blot screening of CFH autoantibodies

Due to the problematic ELISA screen, and research time constrains, IgAN patients were screened against human CFH *via* western blot, as previously described (**Section 5.3.3.2**).

The results of the Western blot screen show CFH IgA autoantibodies present in the plasma of 7 out of 22 IgAN patients (3, 6, 10, 17, 20, 21 and 22), accounting for 31.8% of this cohort. However, only patient 10 and 20 shared an IgA autoantibody response to both bovine and human CFH. However, all 3 BTS controls were CFH IgA autoantibody positive. This suggests the CFH IgA autoantibody response is also present within the normal population. However, conclusions cannot be drawn from this preliminary data regarding the significance of this CFH IgA autoantibody response in IgAN patient plasma.

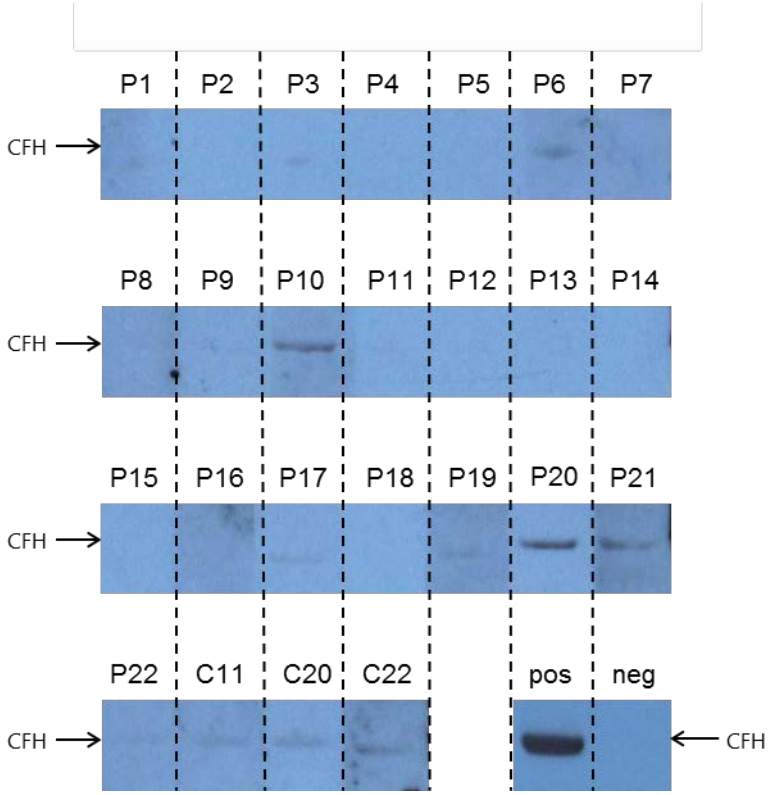


Figure 5.11: Strip blot of IgAN IgA autoantibodies against human CFH

CFH was loaded on 4x 10% SDS-PAGE gels, transferred to nitrocellulose and lanes were cut into a strips. Each strip was incubated with a 1/50 dilution of IgAN plasma (2ml volume), followed by detection with an HRPO-conjugated rabbit anti-human IgA (1/10,000 dilution). IgAN patients are labelled P1-22, and normal controls, C11, C20 and C22. L20/3 (1/2,000) was used as a CFH positive control (pos). A secondary only negative control was also included (neg). The strip blots shown are from film exposed for 10 min.

5.4 Discussion

Autoantibody screening of aHUS plasma did not detect an autoantibody response to CFHR proteins 2-5. Screening against CFHR1 confirmed the presence of 3 autoantibody positive patients. However, all 3 patients had previously been tested and identified as CFH autoantibody positives, with specificity for $CFH_{19\text{-}20}$, and $CFHR1_{4\text{-}5}$ domain fragments. Additionally, each patient was previously determined as homozygous deficient for CFHR1 *via* MLPA and western blot[162]. Therefore, this positivity can be considered as a CFH autoantibody response and not against CFHR1. These data demonstrate that the CFH autoantibody response in aHUS patients is restricted CFH, as no observable cross-reactivity with the CFHR proteins exists. Additionally, the are no patients with a novel CFHR-specific autoantibody phenotype (i.e. CFHR autoantibody positive, but CFH autoantibody negative). We have previously shown the CFH autoantibody response in aHUS primarily targets the C-terminal cell surface recognition domain, and it is this interference that precipitates with aHUS[162]. Therefore, no evidence of CFHR autoantibodies in aHUS suggests the CFHR proteins are not involved the 'cell surface' regulation of complement activation that appears to be key to the pathogenesis of this disease.

While the CFHR autoantibodies do not appear to be involved in aHUS, it is important to consider them in other diseases with a CFH autoantibody phenotype. In particular, MPGN2 patients present with mutations and CFH autoantibodies that predominantly bind the regulatory domain of CFH. As previously described, deficiency of CFH regulatory activity is what drives the progression of CFH-associated MPGN. The CFHR proteins exhibit C3/C5 convertase regulatory activity, and/or C3b binding capability, so their role in the pathogenesis of MPGN should be considered. Therefore, autoantibody screening in these patients is important for future work, particularly CFHR5 as at-risk gene variants are associated with MPGN2[174].

In my analysis of CFHR autoantibodies in aHUS, only IgG autoantibodies were considered, as previously reported CFH autoantibodies were of this isotype[163,178,218,220]. IgM screening could be considered, but this may include detection of 'natural' Abs which not necessarily play a role in pathogenesis. The detection of IgA autoantibodies is also a consideration, especially considering

the role of IgA in IgAN, another form of glomerulopathy, as previously described.

The western blot autoantibody screen of IgAN patients highlighted Ab responses against bovine CFH (n=14), human CFH (n=7), and rCFHR5 (n=2-4). When these results presented, a hypothesis was made regarding bovine CFH positives most likely resulting in a human CFH IgA autoantibody response. However, of the patients tested, only 2 patients (10 and 20) were positive to both species variants of CFH. The human CFH data yielded lower band intensities, relative to the bovine CFH results. Interesting, the human CFH protein is approximately 10x more concentrated than the contaminating bovine CFH (1 µg to 0.1 µg, respectively), yet the IgAN autoantibody response is far greater for bovine CFH.

The disparity between binding to bovine and human CFH was an unexpected result, but it does raise some interesting questions regarding the autoantibody status of IgAN patients. The high bovine CFH signal relative to human CFH, suggests bovine CFH may be a triggering antigen in the development of this response. In triggering an IgA response suggests contact with an antigen that comes into contact with the mucosal immune system, such as a gastrointestinal (GI), urogenital or respiratory infection, or GI food antigen. Sampling of the GI tract by M cells has the potential to trigger an autoimmune response *via* 'molecular mimicry'. As bovine CFH is presented to immune cells (e.g. antigen presenting cells, B-cells) in the lamina propria, a pAb response against bovine CFH could ensure. B-cell receptors with cross-reactivity for epitopes within human CFH are then selected and immunological tolerance is broken. This may also explain why a CFHR5 IgA autoantibody response is present; selection of self-antigen epitopes from a polyclonal B-cell response against a highly homologous xenogeneic protein.

In IgAN patients there is an increase prevalence of coeliac disease when compared to the healthy population, leading to the hypothesis that food antigens may serve as a trigger for IgAN onset and progression[225]. This supports the hypothesis of bovine CFH potentially being involved in disease pathogenesis, at least within a sub-population of IgAN patients.

While several IgAN patients have been identified with an IgA response to CFH and CFHR5, it remains that there is also an antigenic response to these proteins in BTS normal control population. This may suggest the IgA autoantibody response is a naturally occurring Ab found in a percentage of the population. After all, bovine-based food products are a significant part of the western diet. Therefore, it is important to establish whether there is a statistically significant difference in IgA autoantibodies in IgAN patients verses a normal control population.

The CFHR5 IgA autoantibody ELISA screen highlighted 2 positive patients. Further confirmation came from the rCFHR5 IgA autoantibody western blot screen. However, the ELISA screen failed to detect 12 out of the 14 positives, as found in the western blot. This is most likely due to the bovine CFH contaminant being diluted to an undetectable level on the ELISA coating surface. To provide further clarification of these results, both the ELISA and western blot screen of IgAN plasma would benefit from using a CFHR5 sample with a higher level of purity.

As previously described, rCFHR5 purification also led to the purification of a significant amount of bovine CFH in the sample (~20%, as measured *via* densitometry). This level of contamination was not seen until the protein was 'over-loaded' on an SDS-PAGE gel. It appears that the contaminating bovine CFH has been purified with rCFHR5, suggesting the proteins have complexed together in some way. It is unlikely this is a coincidental result, as rCFHR5 was purified from a solution containing a multitude of different bovine serum proteins. Yet, bovine CFH was the selected protein. It is well documented that there is a high degree of sequence conservation in CFH, across mammalian species. Bovine CFH itself has been shown to regulate human complement activation *via* C3b binding[226]. If bovine CFH is able to complex with human CFHR5, its human counterpart may have the capacity to do the same. If there was an interaction between the proteins, this may suggest a dual or support role in a complement regulatory process. This could be investigated in future work.

In summary, there is currently no evidence of a novel CFHR autoantibody process in aHUS patients, suggesting the CFHR proteins are not involved in a complement regulatory process in this disease. However, in a preliminary

cohort of IgAN patients, IgA autoantibodies against CFH and CFHR5 have been identified in ~32% and ~9% patients, respectively. Interestingly, IgA Abs against bovine CFH were identified in >60% of IgAN patients.

Chapter 6

6. Conclusion and Future Work

6.1 Project Summary

During this research project, I have successfully generated a full panel of recombinant human CFHR proteins, produced in a mammalian expression system and purified using HisTag affinity chromatography (**Chapter 3**)

CFHR1, CFHR3 and CFHR4B were selected to produce mAbs, for use to develop ELISA tests to specifically measure CFHR concentration in aHUS and normal control plasma (**Chapter 4**). CFHR-specific mAbs would also be utilised as general reagents within our research group for detection of native and recombinant CFHR proteins. Immunisations with CFHR1 and CFHR4B were completed first due to antigen availability. However, as work progressed, it became clear there would not be sufficient time to complete the CFHR3 mAb production.

I was able to successfully produce 1 x CFHR1 (R1/1037), and 3 x CFHR4 (R4/244, R4/123 and R4/277) mAbs, and fully characterise their function. After specificity testing, R1/1037 showed cross-reactivity for CFHR2, so was deemed unsuitable for use in a sandwich ELISA to specifically detect CFHR1 concentration, unless the standard curve is sufficiently optimised to include ratios of rCFHR1 and rCFHR2. In the plasma test samples, R1/1037 would bind CFHR1 and CFHR2 at an unknown ratio, so comparing this to a CFHR1 only standard curve could deliver spurious and inconsistent results. However, R4/244 and R4/277 showed evidence of specificity for CFHR4. R4/244 was selected and used to initially develop an ELISA for specific detection of CFHR4. Western blot analysis of R4/244 was only detecting CFHR4A, yet CFHR4B was the antigen being used to create the standard curve. This was deemed an inefficient system for the exact quantification of plasma CFHR4A/B, but considered suitable for comparing disease and control group data as the result from each group are relative.

An ELISA was set up using R4/244 to assess if there was a difference in CFHR4 levels between 10 x BTS controls, compared with 10 x aHUS samples,

including three patients with the novel CFHR1/4 deletion[162]. No significant difference was seen between the two groups. Further testing is required on a larger population of plasma samples, the data presented is considered preliminary.

As part of the aforementioned screening methodology, after producing a panel of recombinant CFHR proteins, the proteins were used to screen both aHUS and IgAN patient plasma for the presence of CFHR autoantibodies (**Chapter 5**). In the aHUS cohort, there were no novel findings presented regarding CFHR autoantibodies, apart from the expected CFHR1 autoantibody response in three CFH autoantibody positive patients. As previously described, these patients are *CFHR1* homozygous deficient so should never have been exposed to CFHR1. To have found an autoantibody response targeting CFHR1 only, (no CFH cross-reactivity) was one of the aims of screening a full aHUS cohort, rather than only the CFH autoantibody positive patients.

As there was no evidence of CFHR autoantibodies in the aHUS patient cohort, and it remains unclear whether the CFHR proteins play a role in complement regulation (*in vivo*), either in the kidney, or elsewhere in the body. There have been some recent findings which indicate CFHR5 may be involved in complement regulation in the kidney. CFHR5 nephropathy suggests CFHR5 is involved in complement regulation[197]. *In vitro* analysis shows CFHR5 has both decay-accelerating and cofactor activity[172], and the extended CFHR5 mutant protein correlates with this renal nephropathy[175,227,228]. Additionally, CFHR5 was recently screened in a cohort of aHUS patients, and 3 novel heterozygous missense mutations were discovered associated with the disease[173]. Another set of CFHR5 mutations were also shown in a US aHUS cohort[229]. It appears there is sufficient genetic evidence to implicate CFHR5 variants in disease, but protein functional analysis is required to clarify these findings.

A small cohort of IgAN patients were screened for CFHR autoantibodies. Due to the involvement of IgA Abs in this disease, IgA and IgG detection was investigated. There were no CFHR IgG autoantibodies detected. However, there were some interesting results from the IgA screen against CFHR5. The results from the ELISA were inconsistent with the western blot analysis. However, the western blot data was able to offer a better interpretation of the

autoantibody positive results. Western data (**Figure 5.10**) highlighted IgA autoantibody positivity against CFHR5 in at least 2 of the patients (11 and 16), with 2 others displaying a faint band in the position the CFHR5 dimer (patients 19 and 20). These preliminary data suggest that a selection of IgAN patients have IgA autoantibodies targeting CFHR5. This is sufficient data to test a larger population of IgAN patients for the presence of IgA CFHR5 autoantibodies. However, in these analyses cross-reactivity with contaminating bovine CFH influenced the presentation of results., thus highlighting the need to further purify rCFHR5 from the contaminating elements in the sample. A high resolution chromatography column may be able to separate out these proteins. Alternatively, growing the established CHO/rCFHR5 cell clone in serum-free media would serve as a less expensive method for increasing rCFHR5 purity. It would not be advisable to only screen plasma using western blot, using the contaminated CFHR5 sample, as a secondary mechanism to clarify results is important for consistency and relative accuracy between tests.

From the perspective of future work with CFHR5, it would be interesting to see whether human CFH is able to complex with human CFHR5 in an *in vitro* assay, as suggested by co-purification of rCFHR5 and bovine CFH in the HisTag purification protocol. If this were the case, it may suggest CFH and CFHR5 are working together in some way to regulate complement. As bovine CFH was eliciting a positive IgA autoantibody response, this led to testing the cross-reactivity with human CFH. In ~32% of the IgAN plasma screened, western blot data showing IgA autoantibodies against human CFH. As before, this is sufficient evidence to begin screening a larger population of IgAN patients, and normal controls. CFH screening *via* ELISA proved unsuccessful. For further optimisation, there are now CFH IgA autoantibody positive controls so ELISA parameters can be selected around the upper limits of these positive controls.

Finding CFHR5 and CFH autoantibodies in IgAN, potentially highlights another dimension in the pathogenesis of the disease. If the autoantibodies were blocking the complement regulatory function of CFHR5 and/or CFH, this would help explain why there is scattering of C3 on the mesangial surface. It would be interesting to screen complement genes in IgAN patients, to assess whether there is evidence of a link between the disease and complement dysregulation

at a genomic level. CFH has been screened in a small cohort of patients, but no susceptibility factors were found[100]. However, more recent evidence identified a novel mutation in the CFH N-terminal region to be associated with IgAN. It appears there is a role of CFH and/or CFHR5 in the pathogenesis of IgAN.

The complement regulator CFH was first identified as major risk factor in aHUS in 1983[230]. Since this seminal discovery, our understanding of the complexity of aHUS, and several other disease states has become much clearer and more manageable to present a coherent disease model. CFH is just one component of a much wider community of proteins involved in the complement system, but understanding its involvement in disease has led to the development of a standard methodology for screening aHUS patients for risk-associated features of the disease, including screening for rare and common variants in complement regulatory and activatory genes, genomic rearrangements and autoantibodies. Results have highlighted several other risk-associated genetic variants in other complement regulators (CD46, CFI and TMBD), and complement activators (CFB and C3)[118-120,123]. CFH autoantibodies appear in ~10% of aHUS patients, and correlation with CFHR3/1 (or CFHR1/4) deletion[162]. However, since evidence of a potential autoimmune aspect to aHUS was first published in 2005[218], CFH autoantibodies have also been described in MPGN (type I and II), where the autoantibody response was targeted to the regulatory domain of CFH[231]. We were able to identify CFH autoantibodies in AMD, in association with the *CFHR3/1* deletion, but age-matched controls had a higher mean CFH autoantibody titre[139]. This suggested the *CFHR3/1* deletion was conferring protection against the development of an autoantibody response. More recently, CFH autoantibodies were found associated with rheumatic diseases, SLE and RA[232]. Most interesting is that the autoantibody response in rheumatic disease also presents with a deficiency of *CFHR3/1*. Prior to this study, this had only been observed in aHUS and AMD patients. However, the autoantibody response in these patients was scattered across several epitopes of CFH, compared with C-terminal clustering in aHUS. This suggests that individuals with a *CFHR3/1* deficiency have an increased risk of developing a CFH autoantibody response, but the specificity of Ab binding is not always geared towards the C-terminal region as suggested by aHUS data. Therefore, the CFH autoantibody C-terminal clustering is precipitates with

aHUS. This further implicates the CFH autoantibodies as being involved in a pathogenic process in aHUS, rather than a mere epiphenomenon that develops secondary to other disease-associated factors.

6.2 Future Work

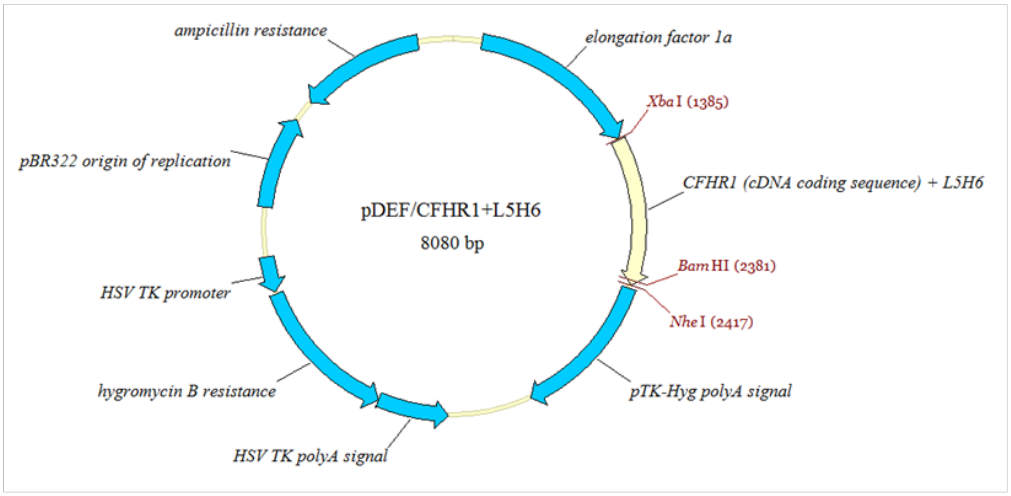
To further establish a robust CFHR-specific detection ELISA, the specificity of the mAb response needs to be driven a protein fragment. The previous methodology aimed to produce hybridomas secreting a selection of Ab clone to target different regions of each protein. However, this process has proved unsuccessful in generating an appropriate CFHR-specific monoclonal. There are limitations to using fragment for immunisation (e.g. restricted antigen profile), but this appears to be the only option to increase the chances achieving targeted specificity for such an assay. Any cross-reactivity is going to interfere with the accuracy of the results. As more disease states are presenting with a CFH autoantibody phenotype, in-conjunction with a CFHR3/1 deletion, the demand for such a test may increase.

While we have CFHR4-specific mAbs, the accuracy of detecting CFHR4 using CFHR4B in the standard curve may present us with an inaccurate measurement of each protein (as previously described). As presentation of the CFHR4/1 deficiency has only been shown in three aHUS patients to-date, it may be more sensible to focus on CFHR1 and CFHR3 for screening to further establish whether CFHR1 deficiency is primarily responsible for triggering the CFH autoantibody presentation. Screening of CFHR proteins in aHUS is complete, so the detection of autoantibodies should now focus on other proteins that are yet to be screened, but have been identified as conferring and increased risk of disease, such as TMBD, CFB and C3.

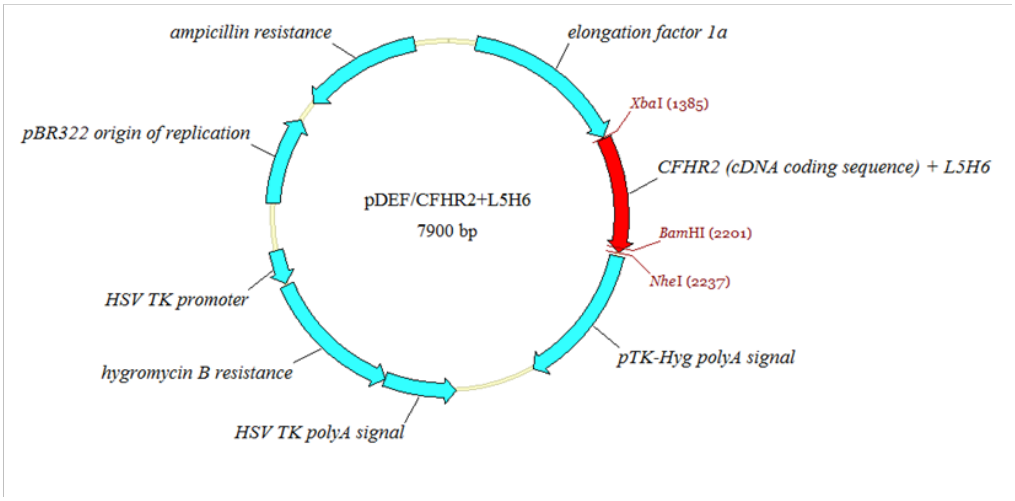
As previously described, a larger cohort of IgAN patients should now be screened against CFH and CFHR5, to assess the frequencies of this autoantibody phenotype between disease and normal controls. This is an essential next step, as ratio of positive results within the population needs careful consideration before association with disease can be attributed.

Appendix I: *pDEF/rCFHR* plasmid constructs

(a)



(b)



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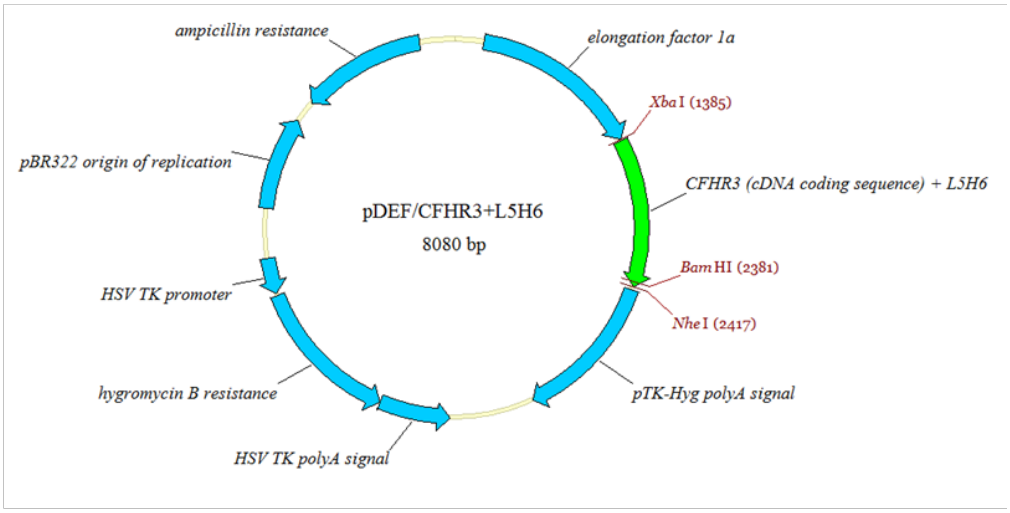
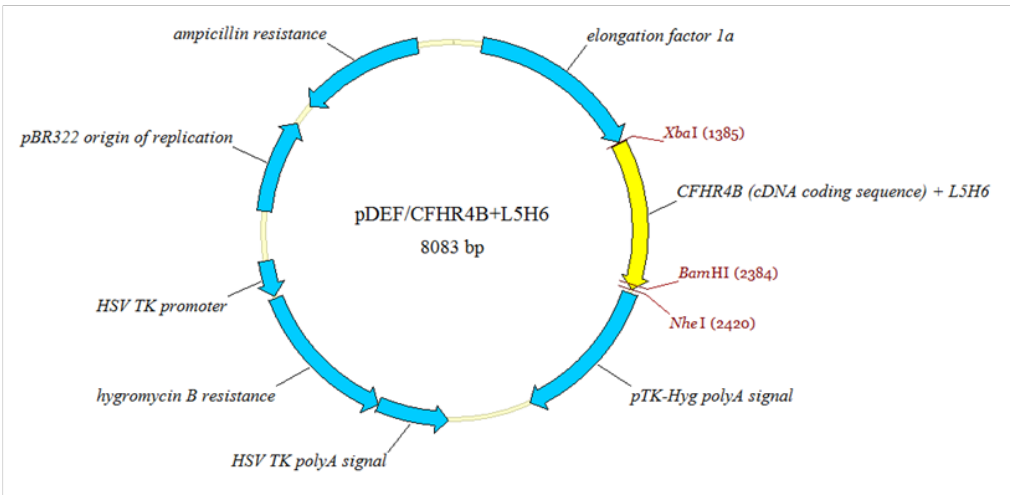

ampicillin resistance
elongation factor 1a
XbaI (1385)
CFHR3 (cDNA coding sequence) + L5H6
BamHI (2381)
NheI (2417)
pTK-Hyg polyA signal
HSV TK polyA signal
hygromycin B resistance
HSV TK promoter
pBR322 origin of replication
pDEF/CFHR3+L5H6
8080 bp

(d)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XbaI (1385)
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BamHI (2384)
NheI (2420)
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hygromycin B resistance
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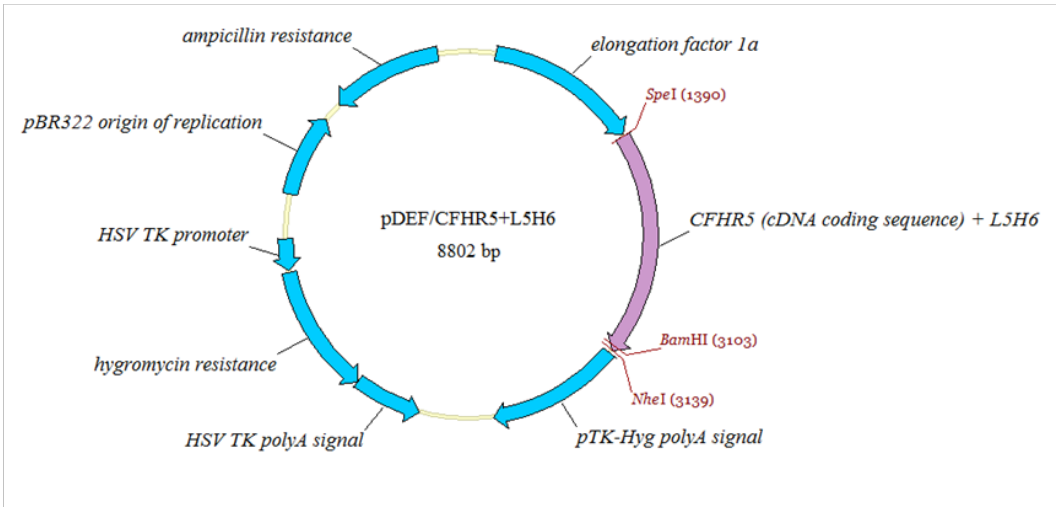
(e)



Appendix I: *pDEF/rCFHR* plasmid constructs

The *pDEF/L5H6* plasmid was used as the base construct to build each *rCFHR* expression plasmid. Each inserted *rCFHR* cDNA was inserted excluding the native stop codon, resulting in the addition of the *L5H6* motif to the C-terminus of each recombinantly expressed CFHR protein. **(a-d)** *rCFHR1-4B* cDNA were inserted using *XbaI* and *BamHI* RE sites. **(e)** *rCFHR5* cDNA was inserted using *SpeI* and *BamHI* RE sites.

Appendix II: mAb and pAbs used

Supplier	Code	Ab
Santa Cruz Biotech	sc-47685	C18/3 (anti-CFH mAb; IgG1)
Santa Cruz Biotech	sc-47686	L20/3 (anti-CFH mAb; IgG1)
AbD serotec	MCA509G	OX24 (anti-CFH mAb; IgG1)
Calbiochem	OB05-100UG	Anti-His•Tag® (mAb; IgG1)
Calbiochem	341276	Goat anti-CFH pAb
Jackson ImmunoResearch	115-035-003	HRPO-conjugated goat anti-mouse IgG (H+L)
Jackson ImmunoResearch	515-035-071	HRPO-conjugated sheep anti-mouse IgG (γ-chain specific)
Jackson ImmunoResearch	115-006-075	HRPO-conjugated goat anti-mouse IgM (μ-chain specific)
Jackson ImmunoResearch	115-006-071	HRPO-conjugated goat anti-mouse IgG (γ-chain specific)
Jackson ImmunoResearch	109-035-008	HRPO-conjugated goat anti-human IgG (γ-chain specific)
Jackson ImmunoResearch	309-035-011	HRPO-conjugated rabbit anti-human IgA (α-chain specific)
Jackson ImmunoResearch	111-035-045	HRPO-conjugated goat anti-rabbit IgG (H+L)

(a) Commercial primary and secondary Abs

Name	Antigen	Cross-reactivity
R1/1037 (mAb; IgG1)	rCFHR1	CFHR2
R4/244 (mAb; IgG1)	rCFHR4A	None
R4/277 (mAb; IgG1)	rCFHR4A/B	None
R4/123 (mAb; IgG1)	rCFHR4A/B	CFH
Rabbit anti-CFHR4B polysera (IgG response)	rCFHR4B	CFH CFHR3 CFHR4A CFHR4B?
Rabbit anti-CFHR5 polysera (IgG response)	rCFHR5	CFH CFHR1 CFHR2 (low level binding) CFHR4A CFHR5

(b) mAb and pAb Abs produced in-house

Appendix III: Presentations and Publications

Presentations

1. European Meeting on Complement in Human Disease; Visegrad, Hungary (5^{th}-8^{th} September 2009). "*Towards a highly specific Factor H ELISA for rapid screening of patient sera*" (poster presentation).

2. ICM Research Day, Newcastle University, Newcastle upon Tyne, UK (23^{rd} June 2010)."Assessing the function of the CFHR proteins in health and disease" (poster presentation).

3. ICM Research Seminar, Newcastle University, Newcastle upon Tyne, UK (6^{th} December 2011). "*Autoantibodies in complement-mediated kidney diseases*" (talk presentation).

Publications:

1. Moore *et al*., (2010) Association of factor H autoantibodies with deletions of CFHR1, CFHR3, CFHR4, and with mutations in CFH, CFI, CD46, and C3 in patients with atypical haemolytic uremic syndrome. *Blood* 115:2;379-387.

2. Dhillon *et al*., (2010) Complement factor H autoantibodies and age-related macular degeneration*. Invest Ophthalmol Vis Sci* 51:11;5858-63.

3. Goodship *et al*., (2012) Factor H autoantibodies in membranoproliferative glomerulonephritis. *Mol Immunol* 52:3-4;200-6.

4. Holmes *et al*., (2012) Determining the population frequency of the CFHR3/CFHR1 deletion in the regulation of complement activation (RCA) gene cluster at 1q32 (*publication under review at time of printing thesis*).

Copies of each publication can be found at the back of this thesis, with notes outlining my contribution to each.

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Publication 1

1. Moore *et al*., (2010) Association of factor H autoantibodies with deletions of CFHR1, CFHR3, CFHR4, and with mutations in CFH, CFI, CD46, and C3 in patients with atypical haemolytic uremic syndrome. *Blood* 115:2;379-387.

Contributions

Figure 3: I conducted the ELISA testing for patient autoantibodies against CFH protein fragments. This data set is shown in my thesis as Figure 5.1.

Figure 4: I conducted the western blots detecting CFH, CFHR1 and CFHR3 in aHUS patients and BTS controls. This figure does not form part of my thesis.

Table 3: I conducted the analysis of MLPA results to determine *CFH/CFHR* copy number the healthy control cohort.

Association of factor H autoantibodies with deletions of *CFHR1*, *CFHR3*, *CFHR4*, and with mutations in *CFH*, *CFI*, *CD46*, and *C3* in patients with atypical hemolytic uremic syndrome

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Factor H autoantibodies have been reported in approximately 10% of patients with atypical hemolytic uremic syndrome (aHUS) and are associated with deficiency of factor H–related proteins 1 and 3. In this study we examined the prevalence of factor H autoantibodies in the Newcastle cohort of aHUS patients, determined whether the presence of such autoantibodies is always associated with deficiency of factor H–related proteins 1 and 3, and examined whether such patients have additional susceptibility factors and/or mutations in the genes encoding complement regulator/activators. We screened 142 patients with aHUS and found factor H autoantibodies in 13 individuals (age 1-11 years). The presence of the autoantibodies was confirmed by Western blotting. By using multiplex ligation-dependent probe amplification we measured complement factor H–related *(CFHR)1* and *CFHR3* copy number. In 10 of the 13 patients there were 0 copies of *CFHR1*, and in 3 patients there were 2. In 3 of the patients with 0 copies of *CFHR1* there was 1 copy of *CFHR3*, and these individuals exhibited a novel deletion incorporating *CFHR1* and *CFHR4*. In 5 patients mutations were identified: 1 in *CFH*, 1 in *CFI*, 1 in *CD46*, and 2 in *C3*. The latter observation emphasizes that multiple concurrent factors may be necessary in individual patients for disease manifestation. (Blood. 2010;115:379-387)

Introduction

It is now well established that atypical hemolytic uremic syndrome (aHUS) is a disease of complement dysregulation.[1] Mutations have been found in the genes encoding both complement regulators (factor H, factor I, and membrane cofactor protein) and complement activators (factor B and C3) in approximately 50% of patients.[2-6] In addition, factor H autoantibodies have been described in a further approximately 10% of patients.[7-9] These antibodies have been shown to block the C-terminal recognition domain of factor H,[8] an area in which it is known that complement factor H (*CFH*) mutations are associated with aHUS cluster.[10] In addition it has been shown that the majority of patients with factor H autoantibodies have associated complete deficiency of factor H–related proteins 1 and 3[9] secondary to a homozygous deletion of the genes (complement factor H–related [*CFHR*]*1* and *CFHR3*) that encode these proteins. This deletion occurs secondary to nonallelic homologous recombination (NAHR) in segmental duplications in the regulators of complement activation gene cluster at chromosome 1q32 and is known to be associated with an increased risk of aHUS,[11] especially in particular subgroups.[12] NAHR is also known to predispose to the formation of a hybrid complement gene associated with aHUS.[13]

Recent studies have shown that patients with aHUS may have mutations in more than one complement regulator/activator, but such mutations have not been described in association with factor H autoantibodies. It is also well established that there are additional susceptibility factors (single nucleotide polymorphisms [SNPs] and haplotype blocks) in *CFH* and *CD46* that increase the risk of developing aHUS.[14] In this study we have therefore examined the prevalence of factor H autoantibodies in the Newcastle cohort of aHUS patients, determined whether the presence of such autoantibodies is always associated with deficiency of factor H–related proteins 1 and 3, and examined whether such patients have additional susceptibility factors and/or mutations in the genes encoding complement regulators/activators.

Methods

Subjects

Patients from the Newcastle cohort of aHUS (n = 308) were included in this study. Serum samples at the time of or shortly after presentation were available from 142 aHUS patients, and of these paired DNA samples were available for 128. Control paired serum and DNA samples were available from 100 local blood donors. The study was approved by the Northern and Yorkshire Multi-Center Research Ethics Committee and informed consent obtained in accordance with the Declaration of Helsinki.

Factor H autoantibody assay

This assay was undertaken in 142 aHUS patients and 100 control subjects. Flexible 96-well plates were coated with 5 μg/mL of purified factor H (Merck Chemicals Ltd) or bovine serum albumin (BSA; Sigma) or molar equivalents of factor H fragments (short consensus repeats [SCRs] 1-4,

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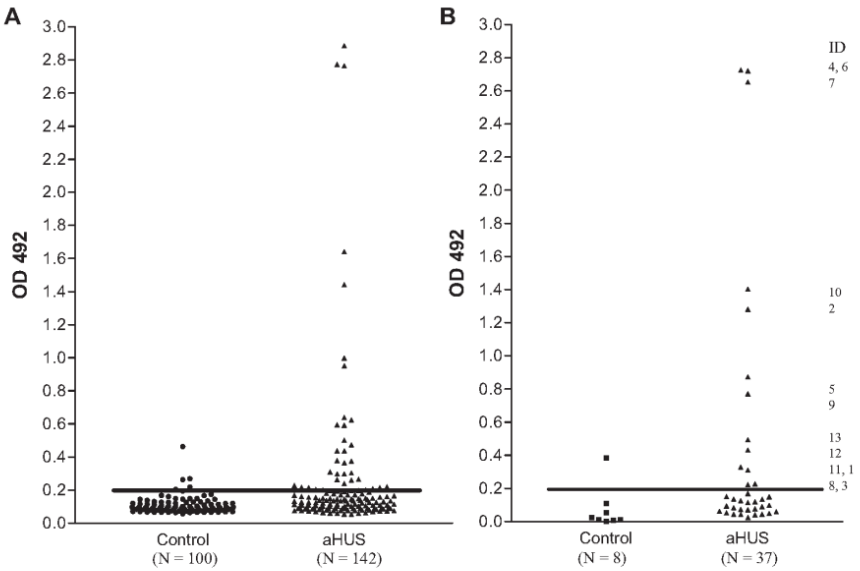

Figure 1. Detection of factor H autoantibodies. Factor H autoantibodies in aHUS patients and control subjects were detected by the use of a sensitive ELISA as described in "Factor H autoantibody assay." (A) OD492 for 100 control subjects and 142 aHUS patients. (B) OD492 for 8 control subjects and 37 aHUS patients with an uncorrected OD value greater than or close to 0.215 (the mean + 2SD for the 100 control subjects) after background reactivity to BSA has been subtracted to exclude false-positive interactions. The identity of the 13 factor H autoantibody–positive patients is shown.

normal dosage. One of each pair of primers was fluorescently labeled (5′FAM), and all primers are shown in Table 2. After PCR, amplification products were analyzed by capillary electrophoresis (ABI, PerkinElmer). Peak areas were obtained for each sample and dosage quotients calculated.

Complement assays

C3 and C4 levels were measured by rate nephelometry (Beckman Array 360). Factor H and factor I levels were measured by radioimmunodiffusion (Binding Site). The normal ranges were C3 (0.68-1.38 g/L), C4 (0.18-0.60 g/L), factor H (0.35-0.59 g/L), and factor I (38-58 mg/L).

Mutation screening and genotyping

In all patients found to have factor H autoantibodies mutation, screening of *CFH*, *CD46*, *CFI*, *CFB*, and *C3* was or had previously been undertaken by the use of direct fluorescent sequencing as described.[2-4,6,19] Genotyping of the following SNPs was undertaken by the use of direct sequencing: *CD46* -652A>G (rs2796267), *CD46* -366A>G (rs2796268), *CD46* c.4070T>C (rs7144), *CFH* -331C>T (rs3753394), *CFH* c.2016A>G p.Gln672Gln (rs3753396), and *CFH* c.2808G>T p.Glu936Asp (rs1065489).

Results

Factor H autoantibodies

In enzyme-linked immunosorbent assays (ELISAs), the mean optical density plus 2SD in the control group before BSA subtraction was 0.215. Eight control patients and 37 aHUS patients had an OD reading greater than or close to this value (Figure 1A). BSA subtraction was undertaken in all these samples. Subsequently 13 patients and 1 control patient had an OD greater than 0.2 (Figure 1B). This cutoff was used to identify the presence of a factor H autoantibody. The specificity of the autoantibodies was confirmed in all 13 patients and the 1 control subject by Western blotting (Figure 2). By the use of a standard strip blot approach, patient sera was exposed to factor H immobilized on nitrocellulose and any factor H–specific human IgG detected. By the use of this approach, autoantibody binding to factor H was demonstrated for all subjects originally identified by ELISA. Signal intensity varied between

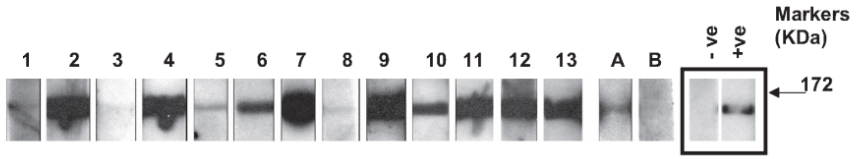

Figure 2. Analysis of antifactor H binding by Western blotting. Purified factor H was run out on 10% SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were then incubated with sera collected from subjects and bound antibody detected as described in "Methods." ECL Western blotting substrate was used to visualize bound antibody. The same secondary reagent and exposure times were used throughout. Sera from known factor H autoantibody–positive and –negative subjects were used to allow standardization across multiple experiments. The positive (+ve) and negative controls (−ve) are on adjacent strips on the same autorad film and are shown as representative control signals (black box). The factor H autoantibody patients (1-13) and 2 normal subjects, A and B, are shown. These data are from a collection of sequential experiments. Molecular weight markers are shown, and the data are representative of at least 3 independent experiments.

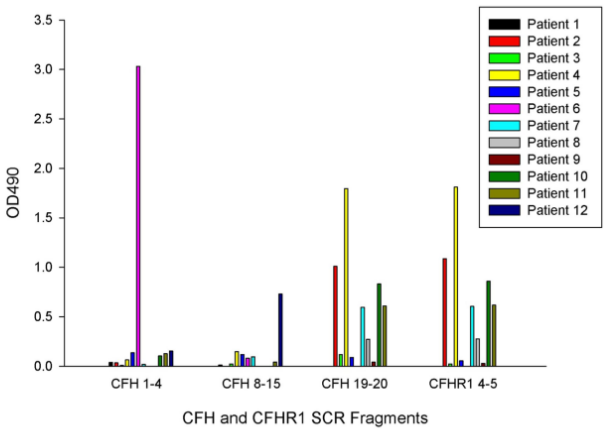

Figure 3. Autoantibody reactivity with short factor H fragments. Autoantibody binding to factor H fragments (corresponding to SCRs 1-4, 8-15, and 19-20) and a factor H–related protein 1 fragment (SCR 4-5) was assessed by the use of ELISA in a similar manner to the original autoantibody screen. Molar equivalent concentrations of the SCR fragments were coated onto separate ELISA plates, and a BSA subtraction was performed. Results are representative of 3 separate experiments.

patients, but in general strong responses via ELISA were mirrored in the western blot analysis.

Binding of autoantibodies to CFH and CFHR1 fragments

The site of binding of the autoantibodies was determined with the use of factor H fragments SCRs 1 to 4, 8 to 15, 19 to 20, and a factor H–related protein 1 fragment SCR 4 to 5 (Figure 3). Antibodies from 1 patient (number 6, *CFHR1* copy number 2) bound only to factor H SCR 1 to 4, from 1 patient (number 12, *CFHR1* copy number 0) they bound only to factor H SCR 8 to 15, and from 7 patients (numbers 2, 3, 4, 7, 8, 10, and 11) they bound to both factor H SCR 19 to 20 and factor H–related protein 1 SCR 4 to 5 in a similar pattern. Of these 7 patients, 6 had 0 copies of *CFHR1*, and 1 (number 7) had 2 copies of *CFHR1*. Three patients (numbers 1, 5, and 9) demonstrated binding to full-length factor H but not to any of these fragments. The control subject with a positive factor H autoantibody demonstrated binding to factor H SCR fragment 8 to 15 with an OD 490 of 1.18.

CFHR1, *CFHR3*, and *CFHR4* copy number

Homozygous deletion of *CFHR1* was significantly more frequent in the aHUS patients than control subjects (aHUS 12.8% vs control 3.0%). Likewise the allele frequency of *CFHR1* deletion was significantly greater in aHUS patients than in control subjects (aHUS 26.5% vs control 17.3%; Tables 3-4). The *CFHR1* deletion frequency in the aHUS patients is not consistent with Hardy-Weinberg equilibrium ($P = 2.8 \times 10^{-4}$). This finding is the result of an excess of homozygous *CFHR1* deletion in the aHUS patients; the frequency of heterozygous *CFHR1* deletion was very similar in aHUS patients and control subjects (aHUS 27.6% vs control 28.7%). In the 13 factor H autoantibody–positive patients, 10 had 0 copies of *CFHR1*. However, 3 patients (6, 7, and 9) had 2 copies of *CFHR1*, as did the 1 control patient who had detectable factor H autoantibodies. This patient's status was confirmed at a protein level by Western blotting (Figure 4). In the 128 aHUS patients with paired serum and DNA samples, there were 6 patients who had no copies of either *CFHR1* or *CFHR3* whose serum samples did not show evidence of factor autoantibodies. In the 10 patients who were positive for factor H autoantibodies and had 0 copies of *CFHR1*, there were 3 (patients 3, 4, and 10) who had a single copy of *CFHR3* rather than none. This finding was confirmed by Western blotting (Figure 4) with the use of factor H–related protein 3 antisera (kindly provided by Professor Peter Zipfel). In these 3 patients we demonstrated that this finding was caused by the presence of a novel deletion (~ 125 kb) in the regulators of complement activation (RCA) cluster that incorporates *CFHR1* and *CFHR4*, leaving *CFHR3* intact (Figure 5).

Using quantitative PCR we showed that these 3 patients (3, 4, and 10) had 1 copy of *CFHR4*, whereas the remaining 10 all had 2 copies of *CFHR4*. Thus, in these 3 patients there was on 1 allele a deletion of *CFHR3/CFHR1* and on the other allele a *CFHR1/CFHR4* deletion. In 143 aHUS patients, copy number of both *CFHR3* and *CFHR1* was measured. In 4 of these patients there was evidence of a heterozygous *CFHR1/CFHR4* deletion, including the 3 aforementioned patients who were found to have factor H autoantibodies. A serum sample was not available for the other patient. In all 4 patients there was evidence of a *CFHR3/CFHR1* deletion on the other allele. Therefore, the allele frequency of the *CFHR1/CFHR4* deletion in aHUS patients is 1.4%. In 505 control subjects there were 9 individuals with evidence of a heterozygous *CFHR1/CFHR4* deletion. In 2 of these there was evidence of a *CFHR3/CFHR1* deletion on the other allele. Therefore, the allele frequency of the *CFHR1/CFHR4* deletion is 0.9% in control subjects. There was no significant difference between aHUS

Table 3. *CFHR1* copy number in aHUS patients and control subjects

CFHR1 copy number	aHUS (n = 196)	Controls (n = 505)
0	25	15
1	54	145
2	115	342
3	2	3

aHUS indicates atypical hemolytic uremic syndrome; and *CFHR1*, complement factor H–related 1.

$\chi^2 = 25.9$; $P = 1.0 \times 10^{-5}$.

Table 4. *CFHR1* allele frequency in aHUS patients and control subjects

CFHR1 allele frequency	aHUS patients (n = 196)	Controls (n = 505)
Deleted	104	175
Present	288	835

aHUS indicates atypical hemolytic uremic syndrome; CFHR1, complement factor H–related 1.

$\chi^2 = 15.0$; $P = 1.1 \times 10^{-4}$.

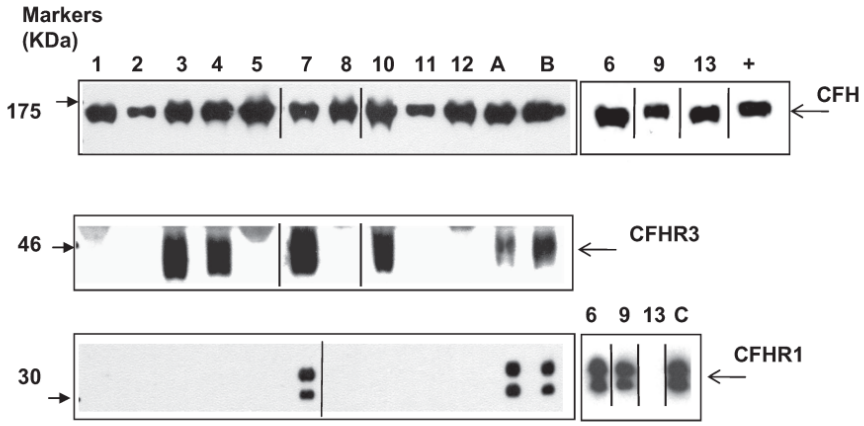

Figure 4. Analysis of factor H, factor H–related protein 3, and factor H–related protein 1 in patients with factor H autoantibodies. Sera from subjects were run out on 10% SDS-PAGE and transferred to nitrocellulose. Factor H, factor H–related protein 3, and factor H–related protein 1 were then detected by staining as described in the Methods section. ECL Western blotting substrate was used to visualize bound antibody. Sera was available from all 13 patients (samples 1-13) for analysis of factor H and factor H–related protein 1, but only from 9 patients for factor H–related protein 3. These 9 samples plus control samples were run on parallel gels. A, B, and C are normal controls known to have 2 copies of *CFHR1* and *CFHR3*. Data from the remaining 3 samples are shown on the right. Purified factor H (equivalent to 0.5 mg/mL) was used as a positive control (+) in the smaller antifactor H blot. Black vertical lines indicate a repositioned gel lane, and black boxes illustrate the individual blots used. This figure is representative of several independent experiments.

patients and control subjects in the allele frequency of the *CFHR1/CFHR4* deletion ($\chi^2 = 1.318$, DF = 1, $P = .251$), but there was insufficient power for us to be certain. However, the combination of a *CFHR3/CFHR1* deletion on one allele and a *CFHR1/CFHR4* deletion on the other was significantly more frequent in the aHUS patients ($\chi^2 = 7.004$, DF = 1, $P = .008$).

Clinical details of the patients with factor H autoantibodies

The clinical details of the 13 patients with factor H autoantibodies are shown in Table 5. There were 6 male and 7 female patients, and the median age at presentation was 8 years (range, 1-11 years). The median length of follow-up was 6 years (range, 1-11 years). Seven patients have regained renal function, 2 of these are on prophylactic long-term plasma exchange, and 1 has had 3 relapses that were successfully treated on each occasion with plasma exchange. Six patients progressed to end-stage renal failure; of these, 3 have received a renal allograft without recurrence in a follow-up period ranging from 2 to 6 years. Levels of C3 were low in 3 patients (numbers 1, 2, and 12) at presentation. One patient (number 5) had a low factor H level, but mutation screening of *CFH* did not show any abnormality. Mutation screening of *CFH*, *CD46*, *CFI*, *CFB*, and *C3* was undertaken in all patients with the use of direct fluorescent sequencing as described previously (Table 6).[2-4,6,19] One patient had a heterozygous sequence variant in *CFH* (c.2850G>T, p.Gln950His), which has been described previously in aHUS but has also been reported by one group in healthy subjects.[21] One patient who has been reported previously had a sequence variant in *CD46* (c.718T>C, Ser240Pro), which has been shown to be functionally significant.[4] A third patient had a previously unreported sequence variant in exon 11 of *CFI* (Exon 11, c.1216C>T, p.Arg406Cys), which is predicted to be functionally significant. Two novel sequence variants were identified in *C3* in 2 patients. One was in the 5′UTR (c.-3_-2dup) and the other in exon 15 (c.1898A>G;p.Lys633Arg). The genotyping results for *CD46* and *CFH* susceptibility factors are shown in Table 7. Ten of the 13 patients were heterozygous for the at-risk G allele of *CD46* -652A>G (rs2796267), and 2 were homozygous. Seven of the 13 patients were heterozygous for the at-risk G allele of *CD46* -366A>G (rs2796268), and 4 were homozygous. Six of the 13 patients were heterozygous for the at-risk C allele of *CD46* c.4070T>C (rs7144), and 4 were homozygous. From these results we inferred that 8 patients (numbers 1, 3, 4, 5, 6, 7, 11, and 12) were heterozygous for the at-risk *CD46* haplotype $CD46_{GGAAC}$, and 2 (numbers 2 and 10) were homozygous. Six patients were heterozygous for the at risk T allele of *CFH* -331C>T (rs3753394), and 2 were homozygous. One patient was homozygous for the at-risk G allele of *CFH* c.2016A>G;p.Gln672Gln (rs3753396), and 1 patient was homozygous for the at-risk allele of *CFH* c.2808G > T;p.Glu936Asp (rs1065489). From these results we inferred that 1 patient (number 6) was homozygous for the at-risk CFH_{TGTGGT} (H3) haplotype.

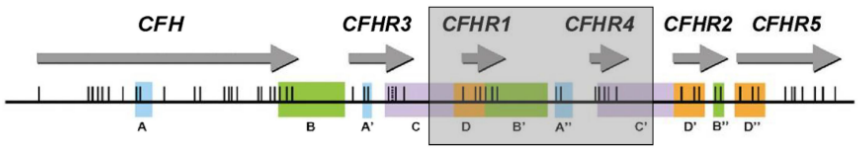

Figure 5. A novel deletion including *CFHR1* and *CFHR4*. Position of the genes encoding factor H and the factor H–related proteins in a centromeric segment of the RCA cluster at 1q32. Regions of high sequence identity (originally determined by Male et al[20]) are indicated by the same letter and color. Exons are indicated as vertical lines. The shaded box shows the presence of a novel ~ 125-kb deletion, which includes *CFHR1* and *CFHR4*. This deletion occurs within the duplicons C/C′ as a result of nonallelic homologous recombination.

Table 5. Clinical details of the aHUS patients with factor H autoantibodies

Patient ID	Age at presentation, y	Sex	Clinical outcome	Length of follow-up, y	Transplanted	C3, g/L	C4, g/L	Factor H, g/L	Factor I, mg/L
1	5	M	Recovered renal function. 2 relapses. On PE 2×/week	2	No	0.61	0.1	0.37	39
2	11	M	Recovered renal function	5	No	0.57	0.15	0.44	70
3	8	F	ESRF	9	No	0.86	0.25	0.49	58
4	4	F	ESRF	1	No	0.89	0.3	0.52	41
5	10	F	ESRF	5	Yes, 3 y no recurrence	0.92	0.2	0.29	n/a
6	8	M	ESRF	6	No	1.08	0.36	0.5	74
7	6	M	ESRF	7	Yes, 2 years no recurrence	0.77	0.28	0.56	77
8	1	M	Recovered renal function	4	No	1.59	0.38	0.67	75
9	9	F	Recovered renal function	11	No	n/a	n/a	0.63	n/a
10	5	M	Recovered renal function; multiple relapses; PE 1×/2 weeks	3	No	1.06	0.13	0.45	68
11	4	F	Recovered renal function; 3 relapses treated with PE	6	No	0.85	0.24	n/a	n/a
12	10	F	ESRF	8	Yes, 6 y no recurrence	0.51	0.23	0.57	62
13	9	F	Recovered renal function	8	No	n/a	n/a	n/a	n/a

aHUS indicates atypical hemolytic uremic syndrome; ESRF, end-stage renal failure; F, female; M, male; n/a, not available; and PE, plasma exchange.

Discussion

In this study we have found that 9.2% (13/142) of the Newcastle cohort of aHUS patients had factor H autoantibodies at the time of or shortly after presentation. This finding corresponds well with previous reports. Dragon-Durey et al[7] screened serum samples from 48 children who presented with aHUS and found factor H autoantibodies in 3 (6.3%). Józsi et al[8] screened plasma samples from 60 patients and found autoantibodies in 5 (8.3%). We have previously shown that deletion of the genes encoding factor H–related proteins 1 and 3 is associated with aHUS.[11] In particular it has been shown that homozygous deletion of *CFHR1* and *CFHR3* is more frequent in aHUS than in control patients. It has recently been shown that the presence of factor H autoantibodies is strongly associated with complete deficiency of factor H–related proteins 1 and 3.[9] Józsi et al[9] showed that in an extended cohort of 147 aHUS patients, 16 (including those described in the previous study by Józsi et al[8]) had factor H autoantibodies. Of these 14 had complete absence of factor H–related proteins 1 and 3 on Western blotting, and 2 had low levels. In their cohort of 147 aHUS patients, there were 22 patients in total with complete absence of factor H–related proteins 1 and 3. Thus, 14 (64%) of 22 patients with complete deficiency of factor H–related proteins 1 had factor H autoantibodies.

This finding agrees well with our cohort, in which 10 (63%) of 16 patients with 0 copies of *CFHR1* had factor H autoantibodies. In Józsi et al's cohort,[8] 14 (88%) of 16 patients with factor H autoantibodies had complete deficiency of both factor H–related proteins 1 and 3. Again this finding agrees well with our cohort, in which 10 (77%) of 13 patients with factor H autoantibodies had 0 copies of *CFHR1*. However, in contrast to Józsi et al's patients with factor H autoantibodies, we found that 3 patients had 2 copies of both *CFHR1* and *CFHR3* with no evidence of a deficiency of

Table 6. Mutation screening of *CFH, CD46, CFI, CFB, C3*, and measurement of *CFHR1, CFHR3*, and *CFHR4* copy number

Patient ID	*CFH*	*CD46*	*CFI*	*CFB*	*C3*	Copy number *CFHR1*	*CFHR3*	*CFHR4*
1	nmd	nmd	nmd	nmd	nmd	0	0	2
2	nmd	nmd	nmd	nmd	c.-3_-2dup	0	0	2
3	nmd	nmd	nmd	nmd	nmd	0	1	1
4	nmd	nmd	Exon 11, c.1216C>T;p.Arg406Cys	nmd	nmd	0	1	1
5	nmd	nmd	nmd	nmd	c.1898A>G;p.Lys633Arg	0	0	2
6	nmd	nmd	nmd	nmd	nmd	2	2	2
7	nmd	nmd	nmd	nmd	nmd	2	2	2
8	nmd	nmd	nmd	nmd	nmd	0	0	2
9	nmd	c.718T>C;p. Ser240Pro	nmd	nmd	nmd	2	2	2
10	nmd	nmd	nmd	nmd	nmd	0	1	1
11	nmd	nmd	nmd	nmd	nmd	0	0	2
12	c.2850G>T; p.Gln950His	nmd	nmd	nmd	nmd	0	0	2
13	nmd	nmd	nmd	nmd	nmd	0	0	2

CFHR indicates complement factor H–related; and nmd, no mutation detected.

Table 7. *CD46* and *CFH* susceptibility factors

Patient ID	*CD46* −652A>G (rs2796267)	*CD46* −366A>G (rs2796268)	*CD46* c.4070T>C (rs7144)	*CFH* −331C>T (rs3753394)	*CFH* c.2016A>G Gln672Gln (rs3753396)	*CFH* c.2808G>T Glu936Asp (rs1065489)
1	AG	GG	CC	CT	AA	GG
2	GG	GG	CC	CC	AA	GG
3	AG	AG	TC	CC	AA	GG
4	AG	AG	TC	CC	AA	GG
5	AG	AG	TC	CC	AA	GG
6	AG	AG	TC	TT	GG	TT
7	AG	AG	TC	CT	AA	GG
8	AG	AA	TT	CC	AA	GG
9	AG	AG	TT	TT	AA	GG
10	GG	GG	CC	CT	AA	GG
11	AG	GG	CC	CT	AA	GG
12	AG	AG	TC	CT	AA	GG
13	AA	AA	TT	CT	AA	GG

factor H–related protein 1 on Western blotting (Figure 4). Two of these patients had the highest autoantibody titers of the whole group. Thus, our data would suggest that a significant autoantibody response to factor H can be mounted in the presence of normal expression of factor H–related proteins 1 and 3.

A novel observation in our study is that in 3 patients the complete deficiency of factor H–related protein 1 is caused by a deletion encompassing *CFHR3* and *CFHR1* on one allele and *CFHR1* and *CFHR4* on the other. This novel deletion (*CFHR1/CFHR4*), like the previously described *CFHR3/1* deletion, has probably occurred as a result of nonallelic homologous recombination through segmental duplications in this region of the RCA cluster (as shown in Figure 5). The allele frequency of this deletion in both our control patients and aHUS patients is low (0.9% vs 1.4%). With the power available to us, there was no significant difference between the 2 groups. However, the presence of this deletion on one allele and the *CFHR3/1* deletion on the other resulting in complete deficiency of factor H–related protein 1 was significantly more common in the aHUS cohort. This finding suggests that it is probably complete deficiency of factor H–related protein 1 that is the significant factor associated with the production of factor H autoantibodies in aHUS. How complete deficiency of factor H–related protein 1 might predispose one to the development of factor H autoantibodies in aHUS is not known. It is possible that it results in a failure of central and/or peripheral tolerance.

Are factor H autoantibodies pathogenic in aHUS? It is well established that *CFH* mutations in aHUS cluster in the C-terminal SCRs and that these mutations are associated with impaired control of complement activation at cell surfaces.[22] Moreover, a transgenic murine model in which the mouse factor H lacks the 5 C terminal SCRs (FHΔ16-20) spontaneously develops aHUS.[23] Józsi et al[8] used recombinant fragments of factor H to map the binding site of factor H autoantibodies in 5 patients. They showed in all 5 that the binding site was in the C-terminal SCRs. In our study we have used a similar approach. In agreement with Józsi et al,[8] we found that autoantibodies from 7 patients showed binding to a fragment comprising SCRs 19 to 20. Thus, most factor H autoantibodies in aHUS bind to and impair the activity of the C-terminal SCRs of factor H. This is the same region in which functionally significant mutations cluster. Moreover, it has been shown that factor H autoantibodies impair the binding of factor H to C3b and are associated with enhanced hemolysis of sheep erythrocytes in patient plasma.[8] In addition we have shown that the autoantibodies from the 7 patients with binding to the factor H fragment SCRs 19 to 20 cross-react with the homologous factor H–related 1 protein fragment SCRs 4 to 5.

Our study is the first to show that in addition to the presence of factor H autoantibodies some patients have mutations in complement genes. In 1 patient we found a *CFH* mutation, in another a *CFI* mutation, in another we had already found and reported a functionally significant *CD46* mutation,[4] and in 2 other patients we found novel *C3* variants. Although the functional significance of some of these sequence variants remains to be established, this finding strengthens the suggestion that multiple concurrent susceptibility factors are necessary in some aHUS patients for the disease to become manifest.[14]

In patients with *CFH*, *CFI*, *CD46*, *CFB*, and *C3* mutations it is now well recognized that additional variants (SNPs and haplotype blocks) in *CFH* and *CD46* act as susceptibility factors for the development of the disease.[24] One particular *CFH* haplotype, CFH_{TGTGGT} (also known as the *CFH*-H3), is associated with an increased risk of aHUS. This haplotype is defined by the following SNPs -331C>**T** (rs3753394), c.184**G**>A Val62Ile (rs800292), c.1204**T**>C p.Tyr402His (rs1061170), c.2016A>**G** p.Gln672Gln (rs3753396), IVS15-543**G**>A intron 15 (rs1410996), and c.2808G>**T** p.Glu936Asp (rs1065489), where the at-risk alleles are in bold. The at-risk haplotype can be tagged by genotyping rs3753394, rs3753396, and rs1065489; we have done this in all 13 patients. This haplotype was present in only 1 patient (number 6) in homozygosity. Of interest, this patient had 2 copies of both *CFHR3* and *CFHR1*. It has recently been established that the *CFHR3/1* deletion is associated with particular *CFH* haplotypes in patients with age-related macular degeneration and control subjects.[25-27] In the study undertaken by Spencer et al,[27] all deletion homozygotes were homozygous for alleles GCGAAG at rs529825, rs2019724, rs1831281, rs6677604, rs3753396, and rs1065489. In our study all the factor H autoantibody–positive patients, who were either homozygous for the *CFHR3/1* deletion or had the *CFHR3/1* deletion on one allele and the *CFHR1/4* deletion on the other allele, were homozygous for alleles "AG" at rs3753396 and rs1065489. Interestingly, 2 patients (numbers 7 and 9) who had 2 copies of *CFHR1* and *CFHR3* were also homozygous for alleles "AG" at rs3753396 and rs1065489. This finding raises the possibility that this particular *CFH* haplotype has a role in the pathogenesis of factor H autoantibodies.

As with *CFH* there is a particular *CD46* haplotype, $CD46_{GGAAC}$, associated with an increased risk of aHUS. This haplotype is defined by the following SNPs -652A>**G** (rs2796267), -366A>**G** (rs2796268), IVS9-78G>**A** (rs1962149), IVS12+638G>**A** (rs859705), and c.4070T>**C** (rs7144). The at-risk haplotype can be tagged by genotyping rs2796267, rs2796268, and rs7144; we have again undertaken this in all 13 factor H autoantibody–positive

patients. We inferred that 8 (numbers 1, 3, 4, 5, 6, 7, 11, and 12) were heterozygous for the at-risk *CD46* haplotype, $CD46_{GGAAC}$, and 2 (numbers 2 and 10) were homozygous. This finding suggests that *CD46* may be acting as an additional susceptibility factor for the development of aHUS in patients with factor H autoantibodies.

The clinical features of the 13 patients with factor H autoantibodies that we describe here are similar to those previously reported. All were children with an age at presentation ranging from 1 to 11 years. Of the 3 patients described by Dragon-Durey et al,[7] only 1 developed end-stage renal failure. Of our 13 patients, 6 developed end-stage renal failure, and 7 have maintained renal function to date. Three of the patients who developed end-stage renal failure have undergone transplantation without recurrence of the disease, with a follow-up period ranging from 2 to 6 years. A recent report documented the pretransplant administration of the anti-CD20 monoclonal antibody rituximab followed by intensive plasma exchange postoperatively in a 10-year-old girl with factor H autoantibodies.[28] Before the administration of rituximab, the patient had been treated with prednisolone, azathioprine, and plasma exchange in an attempt to decrease the autoantibody titer. However, this was not achieved until after the administration of rituximab. Subsequently there was no recurrence of the disease after transplant, with a follow-up period of 2 years. We do not have serum samples available from the immediate pretransplant period from patients 5, 7, and 12 to determine autoantibody titer at that time, but we have analyzed a recent convalescent sample from patient 12. This sample was negative for factor H autoantibodies. This finding suggests that the titer of factor H autoantibodies may spontaneously decline with time. A pragmatic approach to the transplant management of patients with factor H autoantibodies would be to regularly monitor antibody levels and administer rituximab to those with persistently elevated titers.

In summary we have confirmed that factor H autoantibodies are found in approximately 9% of patients with aHUS. While most have complete deficiency of factor H–related protein 1, we have shown that this is not a prerequisite. Moreover, we have shown that deficiency of factor H–related protein 1 can arise from a novel *CFHR1/4* deletion and that mutations of *CFH*, *CD46*, *CFI*, and *C3* are present in some patients. The latter observation provides further evidence that multiple "hits" are necessary in some patients before aHUS presents clinically.

Acknowledgments

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Authorship

Contribution: T.H.J.G. and K.J.M. designed the research, analyzed the data, and wrote the paper; and I.M., L.S., I.P., D.K., P.N.B., A.P.H., C.Q.S., S.J.S., L.V.H., R.W., L.M., and K.J.M. performed the research.

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Publication 2

2. Dhillon *et al*., (2010) Complement factor H autoantibodies and age-related macular degeneration*. Invest Ophthalmol Vis Sci* 51:11;5858-63.

Contributions

Table 3: I conducted the analysis of MLPA results to determine *CFH/CFHR* copy number the healthy control cohort. This data set is not included in this thesis.

Complement Factor H Autoantibodies and Age-Related Macular Degeneration

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Purpose. In this case-control study, the hypothesis that factor H autoantibodies are associated with age-related macular degeneration (AMD) was examined.

Methods. One hundred AMD patients (median age, 78 years), 98 age-matched control subjects (median age, 78 years) known not to have AMD, and 100 healthy blood donors (median age, 43 years) were enrolled. An enzyme-linked immunosorbent assay (ELISA) was used to screen for complement factor H autoantibodies and either quantitative polymerase chain reaction (qPCR) or multiplex ligation–dependent probe amplification (MLPA) were performed to measure the copy number of the gene encoding complement factor H–related protein 3 (*CFHR3*).

Results. There was a significant difference in the median complement factor H autoantibody titer between the three groups (AMD patients, 196 reference units [RU]]; age-match control subjects, 316 RU; and blood donor control subjects, 121 RU; Kruskal-Wallis test, $P < 0.001$). Pair-wise comparison (Mann-Whitney test) showed that all three groups were significantly different from each other. Two different thresholds were used in the healthy blood donors to identify individuals with complement factor H autoantibodies. Both suggested that the prevalence of factor H autoantibodies was decreased in AMD patients. The *CFHR3* copy number was measured as a surrogate for the deletion of the genes encoding complement factor H–related proteins 3 and 1 (*CFHR3/1*). The allele frequency of the deletion was significantly higher in the age-matched control subjects than in the AMD patients (22.2% vs. 8.2%).

Conclusions. The level of factor H autoantibodies is lower in AMD patients than in age-matched control subjects. (*Invest Ophthalmol Vis Sci.* 2010;51:5858–5863) DOI:10.1167/iovs.09-5124

It is well accepted that naturally occurring variability in the genes encoding both regulators and activators of the complement system are associated with susceptibility to age-related macular degeneration (AMD). The complement system can be activated by three routes: the classic, the lectin, and the alternative pathways.[1] All three of these pathways lead to generation of the pivotal molecule C3b. Accumulation of undesirable quantities of C3b is avoided by the synthesis of regulatory proteins by the host that inhibit C3b formation from C3, both on cell surfaces and in plasma. They include the serum protein factor H and transmembrane regulators, such as membrane cofactor protein, decay-accelerating factor, and complement receptor 1. A series of studies published in 2005 identified a sequence variant (c.1277T>C; p.Tyr402His, rs1061170) in the gene encoding complement factor H (*CFH*) as a major susceptibility factor for AMD.[2–5] The Tyr402His variant of factor H lies in the seventh short consensus repeat (SCR). Since then, both SNPs and haplotype blocks in other complement genes, including those encoding factor B (*CFB*), factor I (*CFI*), *C2*, and *C3* have been shown to be associated with AMD.[6–10] In addition, a deletion in the RCA cluster that leads to loss of the genes encoding complement factor H–related proteins 1 and 3 (*CFHR3*, *CFHR1*) is associated with protection against the development of AMD.[11,12] Other risk factors for AMD besides age and complement include smoking, racial background, obesity, and sequence variants in the *ARMS2/HTRA1* region on chromosome 10.[13,14]

It is also well established that complement dysregulation predisposes to the development of the renal disease atypical hemolytic uremic syndrome (aHUS). Mutations have been found in the genes encoding both complement regulators (*CFH*, *CFI*, and *MCP*) and complement activators (*CFB* and *C3*) in ~50% of patients.[15] In addition factor H autoantibodies have been described in a further ~10% of patients.[16–18] These antibodies have been shown to block the C-terminal recognition domain of complement factor H,[17] an area where it is known that *CFH* mutations associated with aHUS cluster.[19] Moreover, it has been shown that most patients with factor H autoantibodies have a complete deficiency of complement factor H–related proteins 1 and 3,[18] secondary to the aforementioned deletion that is associated with protection against

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AMD. This deletion occurs as a result of nonallelic homologous recombination within segmental duplications in the regulators of complement activation gene cluster on the long arm of chromosome 1, region 32.

The observation that a high percentage of patients with dense deposit disease (DDD, also known as type II membranoproliferative glomerulonephritis [MPGN]) have drusen led to the identification of the aforementioned sequence variant in *CFH* as a susceptibility factor for AMD.[20] To date, there has been one report of a complement factor H autoantibody in association with MPGN.[21] The Tyr420His variant of factor H lies in SCR 7 of complement factor H, an area known to have a role in binding to glycosaminoglycans (GAGs) and possibly CRP on cell surfaces. It has been shown that the affinities of the two allelic variants for GAGs are significantly different,[22,23] consistent with a local structural difference in GAG-binding sites.[24] Similar results for the binding to CRP have been reported,[25] but whether this is physiological is uncertain.[26–28] Antibodies binding specifically to this region may therefore have a similar modulator effect on ligand binding and predispose to AMD. This forms the basis of the hypothesis that we tested in the present study. To examine this hypothesis, we screened for complement factor H autoantibodies in a cohort of AMD patients and two control samples: one age-matched and the other derived from blood donors.

Methods

Subjects

Plasma and DNA samples were available from 100 patients with AMD, 98 age-matched normal control subjects known not to have AMD, and 100 healthy blood donors (blood donor control subjects). The AMD patients and age-matched control subjects are a subgroup of a larger cohort that has been reported previously.[8] The individuals within this subgroup were chosen at random from the larger cohort, and the control subjects were age and sex matched. The AMD patients were recruited between 2004 and 2006 from ophthalmic clinics in Dundee, Inverness, and the Lothian region of Scotland. The age-matched control subjects were recruited from the same sources and included spouses, subjects who had undergone cataract surgery, and the Lothian birth cohort.[29] The subjects were examined by an ophthalmologist, and data were collected regarding medical history, lifestyle, and smoking history. Color, stereoscopic fundus photography of the macular region was performed in all subjects. A study investigator graded images; for validation, images from 100 case subjects and control subjects were independently graded at the Moorfields Reading Centre ($\kappa = 0.84$). Written informed consent was obtained from all subjects. The research protocol was in accordance with the provisions of the Declaration of Helsinki. Approval for the study was obtained from a multicenter research ethics committee (MREC/03/0/41).

Complement Factor H Autoantibody Assay

This assay was undertaken in all three groups. Flexible, 96-well plates were coated with 5 μg/mL of purified complement factor H (Merck Chemicals, Ltd., Nottingham, UK) or molar equivalents of complement factor H fragments (short consensus repeats [SCRs] 1–4, 6–8, 8–15, and 19–20)[30,31] or molar equivalents of a complement factor H–related protein 1 fragment (SCRs 4–5)[31] in pH 7.6 coating buffer (AbDserotec, Kidlington, UK) and incubated overnight at 4°C. The plates were then washed three times with PBS/Tween 0.01%, followed by blocking for 45 minutes (200 μL per well of Ultrablock; AbDserotec) at room temperature. At this point, a duplicate plate was set up with a blocking solution to act as a background binding control. After blocking, a 1:50 dilution of sera in PBS/Tween 0.01% was loaded (50 μL) in triplicate onto both plates and incubated for 1 to 2 hours. The plates were washed three times and then blocked again. Goat anti-human IgG horse radish peroxidase (HRP; Stratech Scientific, Newmarket, UK) at 1:4000 was added and incubated for 1 hour at room temperature. The plates were washed twice with PBS/Tween 0.01% and twice with PBS. Tetramethyl benzidine (TMB) readymade standard kinetic solution (AbDserotec) was added to each well for 7 minutes exactly, before the reaction was stopped with 10% sulfuric acid. Absorbance at an optical density of 450 nm (OD_{450}) was established with a plate reader (SpectraMax 190; MDS Analytical Technologies, Ltd., Coventry, UK). Triplicate data were analyzed, and mean blocking agent–only readings (Ultrablock; AbDserotec) were subtracted from the mean complement factor H readings, to control for nonspecific/false-positive readings. Purified complement factor H was batch tested for reactivity with anti-human IgG antibody before use in the ELISA. For the anti-factor H assay, a standard curve was generated from a known positive (a kind gift from Marie-Agnes Dragon-Durey, Hôpital Européen Georges Pompidou, Paris, France) and the OD_{450} value for the 1:50 dilution given an arbitrary 10,000 relative units. Linear-regression curve fit analysis was preformed, and reference units (RU) were calculated for each sample accordingly (Prism, ver. 3; GraphPad, San Diego, CA).

Measurement of *CFHR3* Copy Number

The *CFHR3* copy number was measured to determine the allele frequency of the *CFHR3/1* deletion in all three groups. In the AMD and age-matched control subjects, the *CFHR3* copy number was measured using quantitative polymerase chain reaction (qPCR). Two sets of primers and minor groove binder (MGB) probes (Applied Biosystems [ABI], Warrington, UK) were designed on computer (Primer Express ver. 2.0; ABI). Each set consisted of a pair of primers and a 3′-fluorescent-tagged probe (Table 1). One pair of primers and a fluorescein amidite (FAM) 5′-labeled probe were designed inside intron 3 of *CFHR3*. Another pair of primers and a 5′-labeled probe (VIC; ABI) was designed inside the β-globin (*HBB*) gene and used as an endogenous control. Each qPCR was performed in triplicate using 384-well optical-reaction plates (ABI). Five-microliter reactions contained 1 μL of DNA (10 ng/μL) used as template, 2.5 μL of 2× PCR master mix (TaqMan Universal; ABI), 0.2 μL of each primer at 10 μM, 0.2 μL of each probe at 1 μM, and 0.3 μL of dH_2O (Invitrogen-Gibco; Paisley, Scotland, UK). Reactions were performed in real time, with the absolute quantitation (standard curve) setting on a real-time PCR system (HT7900; ABI). Conditions used in the qPCR were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. After completion of PCR, fluorescence was read by using the system software (SDS; ABI), and the resulting cycle thresholds (Ct) were exported to a spreadsheet and analyzed (Excel; Microsoft Redmond, WA).

In the blood donor control subjects, the *CFHR3* copy number was measured with multiplex ligation-dependent probe amplification[32] (MLPA; SALSA MLPA kit, P236-A1 ARMD; MRC Holland). In this kit are six probes for *CFHR3*. Details of these are given in Table 2.

Statistics

Values for antibody titer within the three groups are expressed as the median (range). Comparisons between groups were made using the nonparametric Kruskal-Wallis and Mann-Whitney Tests. The allele fre-

TABLE 1. Primer and Probe Sequences for the CFHR3 qPCR Dosage Analysis

Name	Primer Sequence (5′–3′)	5′ Fluorescent Dye
CFHR3-F	TGGGCATTAGTCAAGAATACAGTAAAA	—
CFHR3-R	ATTAATGCCGCTTCAATATGACTTT	—
CFHR3-Probe	AATTAGAACACAATACTTGTTGGC	6-FAM
Bglobin-F	GGGCAGAGCCATCTATTGCTT	—
Bglobin-R	TGGTGTCTGTTTGAGGTTGCTAGT	—
Bglobin-Probe	TTGCTTCTGACACAACTG	6-VIC

TABLE 2. MLPA Probes Used to Determine *CFHR3* Copy Number

Gene, Exon	Ligation Site	Partial Sequence (20 Nucleotides Adjacent to Ligation Site)
CFHR3, Exon 1	Intron 1	AGGTAAGTTA-AAAGAGATCT
CFHR3, Exon 2	Intron 1	CATTTTCTTG-TGGAATTACA
CFHR3, Exon 3	Intron 3	CGGACGACAG-TCTCAGACTT
CFHR3, Exon 4	Intron 4	GGGTTATATG-AATTCCTACA
CFHR3, Exon 6	Intron 5	TTCCCCAACA-TCACAGCAGA
CFHR3, Exon 6	1003–1002 reverse	TCCCTTCCCG-ACACACTGCT

quency of the *CFHR3/1* deletion in the three groups was compared by using the χ^2 test.

RESULTS

Subjects

The median age of the AMD patients was 78 years (range, 53–96; 38 men and 62 women); that of the age-matched normal control subjects was 78 years (range, 48–92; 38 men and 60 women); and that of the healthy blood donors was 43 years (range, 18–68; 44 men and 56 women).

Of the AMD patients, 19 had severe non-neovascular (dry), and 81 had severe neovascular (wet) changes. There was no significant difference in smoking history between the AMD patients and the age-matched control subjects.

Complement Factor H Autoantibodies

In ELISAs, the median (range) antibody titer (in RU) in the three groups was AMD patients, 196 (0–1495); age-matched control subjects, 316 (0–1743); and blood donor control subjects, 121 (0–3104) (Fig. 1; Kruskal-Wallis Test $P < 0.001$). Pairwise comparisons by Mann-Whitney test showed that all three groups were significantly different from one another (AMD patients versus blood donor control subjects, $P < 0.05$; AMD patients versus age-matched control subjects, $P < 0.01$; age-matched control subjects versus blood donor control subjects, $P < 0.001$). In the three groups, there was no significant difference in median antibody titer between the men and women (AMD patients, male 190 vs. female 206; age-matched control subjects, male 317 vs. female 316; blood donor control subjects, male 123 vs. female 108). The relationship between autoantibody titer and age is shown as a composite scattergram (Fig. 2). Although this result suggests that the prevalence of factor H autoantibodies increases with age, there was no relationship between age and autoantibody titer in the individual groups (AMD patients, $r^2 = 0.090$, $P = 0.375$; age-matched control subjects, $r^2 = -0.086$, $P = 0.401$; blood donor control subjects, $r^2 = 0.034$, $P = 0.739$). The threshold for determining autoantibody positivity in the AMD patients was calculated in two ways. First, we used the mean antibody titer +2SD from the blood donor control subjects. This is the method that most groups,[16,17] including our own,[33] have used, but it does not take into account the non-normal distribution. The mean antibody titer +2SD in the blood donor control subjects was 810 RU, and accounting for individual sample variance, a value over 900 RU was taken as indicative of the presence of a complement factor H autoantibody. In both the AMD patients and the age-matched control subjects, there were eight individuals with an autoantibody titer greater than this threshold. In the blood donor control group, there was only one. Second, to take into account the non-normal distribution of antibody titer in all three groups, we used the 0.975 fractile, as recommended by the International Federation of Clinical Chemistry,[34] of the blood donor control subjects and derived a threshold of 624 RU. With this threshold, there would be 21 age-matched control subjects and 10 AMD patients who were autoantibody positive. This frequency is significantly different between the two groups ($\chi^2 = 4.895$, $df = 1$, $P = 0.027$). The two thresholds are shown in Figure 1.

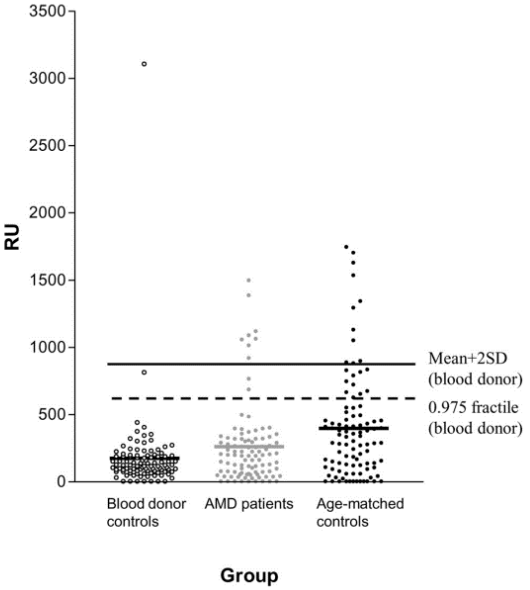

FIGURE 1. Complement factor H autoantibody titer in blood donor control subjects, AMD patients, and age-matched control subjects. *Horizontal solid line*: the threshold of 900 RU, which was derived from the mean +2 SD of the autoantibody titer in the blood donor control subjects. *Dashed line*: threshold of 624 RU which represents the 0.975 fractile of the blood donor control subjects.

Binding of Autoantibodies to CFH and CFHR1 Fragments

The binding sites of the autoantibodies were determined by using complement factor H fragment SCRs 1 to 4, 6 to 8, 8

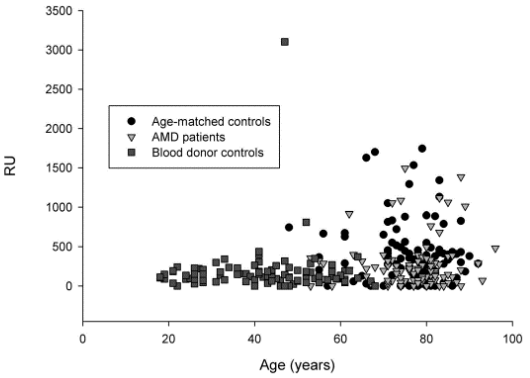

FIGURE 2. Complement factor H autoantibody titer versus age for the three groups: AMD patients $r^2 = 0.090$, $P = 0.375$; age-matched control subjects $r^2 = -0.086$, $P = 0.401$; and blood donor control subjects $r^2 = 0.034$, $P = 0.739$.

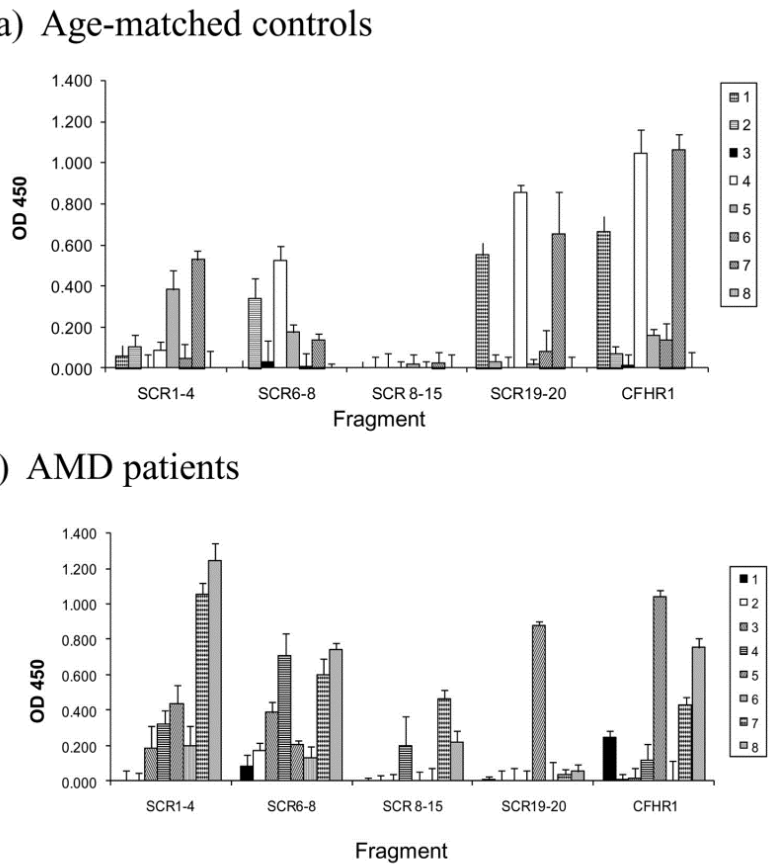

FIGURE 3. Autoantibody reactivity with short factor H fragments. Autoantibody binding (represented as OD_{450}) to factor H fragments (corresponding to SCRs 1–4, 6–8, 8–15, and 19/20) and a factor H–related protein 1 fragment (SCRs 4/5) was assessed with ELISA. Data are expressed as the mean ± SD of results in three experiments. (**a**) Results of the age-matched control subjects; (**b**) AMD patients. Each bar represents one subject.

to 15, and 19/20 and a complement factor H–related protein 1 fragment SCR 4/5 (Fig. 3). There was evidence of binding by autoantibodies from both the AMD patient group and the age-matched control subjects to all these fragments, with the exception of complement factor H SCRs 8 to 15, which was not recognized by the age-matched control subjects. There was a bias in the AMD patients (7/8) for stronger autoantibody reactivity against SCRs 1 to 8 of complement factor H compared with the age-matched control subjects (2/8), but given the overall spectrum of binding and the level of interaction, it is unlikely to be significant.

CFHR3 Copy Number

The *CFHR3* copy number was used as a marker of the *CFHR3/1* deletion. Homozygous deletion of *CFHR3/1* was significantly more frequent in the age-matched control subjects than in the AMD patients (age-matched control subjects 5.6% vs. AMD patients, 0%; $\chi^2 = 14.3$, $df = 2$, $P = 8.0 \times 10^{-4}$). The frequency of homozygous deletion of *CFHR3/1* in the blood donor control subjects was 2%, which was not significantly different from either the AMD patients or the age-matched control subjects. Likewise, the allele frequency of the *CFHR3/1* deletion was significantly greater in age-matched control subjects than in the AMD patients (age-matched control subjects 22.2% vs. AMD patients, 8.2%; $\chi^2 = 14.6$, $df = 1$, $P = 1.3 \times 10^{-4}$; Table 3). The allele frequency of the *CFHR3/1* deletion was also significantly greater in the blood donor control subjects than in the AMD patients (blood donor control subjects 15.5% vs. AMD patients, 8.2%; $\chi^2 = 5.1$, $df = 1$, $P = 0.024$). The *CFHR3/1* deletion frequency in all three groups is consistent with Hardy-Weinberg equilibrium.

TABLE 3. *CFHR3* Copy Number and Allele Frequency

	AMD Patients ($n = 98$)	Age-Matched Control ($n = 90$)	Blood Donor Control ($n = 100$)
CFHR3 copy number			
0	0	5	2
1	16	30	27
2	82	55	71
CFHR3 allele frequency*			
Deleted	16 (8.2%)	40 (22.2%)	31 (15.5%)
Present	180	140	169

* AMD patients vs. age-matched controls; $\chi^2 = 14.6$, $df = 1$, $P = 1.3 \times 10^{-4}$; AMD patients vs. blood donor controls $\chi^2 = 5.1$, $df = 1$, $P = 0.024$; age-matched controls vs. blood donor controls $\chi^2 = 2.818$, $df = 1$, $P = 0.093$.

CFHR3/1 Deletion and Complement Factor H Autoantibodies

There were no AMD patients with the homozygous *CFHR3/1* deletion. As reported, there were eight AMD patients, eight age-matched control subjects, and one blood donor control with complement factor H autoantibodies. Of these 17 individuals, 13 had two copies of *CFHR3* and 4 had one copy (3 age-matched control subjects and 1 AMD patient). Figure 4 shows the antibody titer for each of the groups, according to *CFHR3* copy number. There was no evidence of an association between *CFHR3* copy number and autoantibody titer in the three groups (Kruskal-Wallis test: AMD patients, $P = 0.893$; age-matched control subjects, $P = 0.123$; blood donor control subjects, $P = 0.360$) (Table 4, Fig. 4).

Discussion

In this study, we examined the hypothesis that complement factor H autoantibodies are associated with AMD. We found, by using a threshold derived from the mean +2SD autoantibody of the blood donor control subjects, that complement factor H autoantibodies were present in 1 (1%) of 100 blood donor control subjects, 8 (8%) of 100 AMD patients, and 8 (8.2%) of 98 age-matched control subjects. Using a lower threshold derived from the 0.975 fractile of the blood donor control subjects, we found that 21 (21%) age-matched control subjects and 10 (10%) AMD patients were autoantibody positive. The hypothesis that we set out to test in this study was that the prevalence of factor H autoantibodies is increased in AMD patients. However, the results showed the opposite, in that the complement factor H autoantibody titer was significantly higher in the age-matched control subjects, and the prevalence of autoantibody positivity was significantly greater in the age-matched control subjects when we used the lower 0.975 fractile blood donor threshold. We believe that it would be tenuous to suggest from this that factor H autoantibodies protect against the development of AMD. However, the observation is fascinating. We have confirmed again that the deficiency of factor H–related protein 1 is associated with a decreased risk of AMD, and we speculate that this protein may have additional immunomodulatory properties.

Although determining the relation of age to the presence of factor H autoantibodies was not a goal of this study, the results suggest that the prevalence of factor H autoantibodies was greater in the older age groups. The median age of the blood donor control subjects (48 years) was substantially less than that of the AMD patients and age-matched control subjects (both 78 years), and the complement factor H autoantibody titer was significantly lower in the blood donor control subjects than in either the age-matched control subjects or the AMD patients. However, within each group there was no association between age and autoantibody titer. To test the hypothesis that the prevalence of factor H autoantibodies increases with age necessitates a prospective study, but it has been known for many years that the prevalence of both organ-specific and systemic antibodies increases with age.[35] Factors that may predispose to this phenomenon include immune senescence involving a decline in naïve T cells with a compensatory accumulation of memory T cells, thymic atrophy, chronic inflammation, and age-associated changes in epigenetic phenomenon.[36,37] Whether such autoantibodies are disease-predisposing or disease-causing is uncertain.

Table 4. Factor H Autoantibody Titer

CFHR3 Copy Number	AMD Patients	Age-Matched Control	Blood Donor Control
0		560	219
1	166	276	138
2	202	354	114

Data are in median reference units (RU). Kruskal-Wallis test: AMD patients $P = 0.893$, age-matched control $P = 0.123$, blood donor control $P = 0.360$.

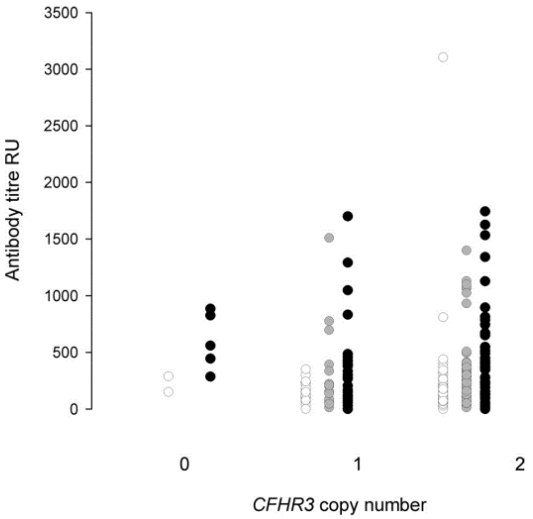

Figure 4. Factor H autoantibody titer for each of the three groups according to *CFHR3* copy number. (○), Blood donor control subjects, (◍), AMD patients, (●), age-matched control subjects.

As in other studies, we have shown that deletion of *CFHR3* and *CFHR1* is associated with a lower prevalence of AMD.[11,12,38] Complement factor H-related protein 1 lacks the regulatory domain of factor H but has been shown to inhibit C5 convertase activity.[39] The C-terminal region of complement factor H-related protein 1 (SCRs 3, 4, and 5) has a high degree of homology with factor H (SCRs 18, 19, and 20) and thus can compete with complement factor H for the same binding sites on cell surfaces.[40] The same deletion has been shown to be associated with complement factor H autoantibodies in aHUS.[16–18,33] The autoantibodies in most such patients bind to SCRs 19 and 20 of complement factor H and thus block the recognition domain.[17] In this study, we have examined both the relationship of complement factor H autoantibodies to the *CFHR3/1* deletion and mapped their binding sites. In the 17 individuals found in this study to be positive for complement factor H autoantibodies (using the higher threshold of mean +2SD), 13 had two copies of *CFHR3* and 4 had one copy, suggesting that only 4 of the 17 carried the *CFHR3/1* deletion. These autoantibodies were at low levels compared with most of those found to be associated with aHUS. We also found evidence of binding to multiple segments of the complement factor H molecule, indicative of a polyclonal antibody response in contrast to the limited clonality seen in aHUS.

In summary, we have found that the prevalence of factor H autoantibodies is decreased in AMD patients compared with age-matched control subjects.

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Publication 3

3. Goodship *et al*., (2012) Factor H autoantibodies in membranoproliferative glomerulonephritis. *Mol Immunol* 52:3-4;200-6.

Contributions

Figure 2: I conducted the western blots detecting CFH, CFHR1 and CFHR3 in MPGN patients and BTS controls. This figure does not form part of my thesis.

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Factor H autoantibodies in membranoproliferative glomerulonephritis

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ABSTRACT

Factor H autoantibodies are found in ~10% of aHUS patients. Most are associated with complete deficiency of factor H related proteins 1/3 and bind to the C terminal recognition domain. MPGN, like aHUS, is characterised by complement activation. In this study we, therefore, examined the hypothesis that factor H autoantibodies are associated with MPGN. We screened sera from 16 MPGN patients and 100 normal controls using ELISA and detected strongly positive IgG factor H autoantibodies in 2 patients. One patient had type II (DDD) MPGN (male aged 24 yrs) with C3NeF and the other type I (female aged 26 yrs) with no detectable C3NeF. We identified the binding site of the autoantibodies using small SCR domain fragments in the ELISA and showed that the autoantibodies in both patients bound predominately to the N terminal complement regulatory domain of factor H. We measured *CFHR 1/3* copy number using MLPA and showed that both patients had 2 copies of *CFHR1* and 3. Finally, we examined the functionality of detected factor H autoantibodies using purified patient IgG and observed increased haemolysis when purified IgG from both patients was added to normal human sera prior to incubation with rabbit red blood cells. Thus, in a cohort of MPGN patients we have found a high titre of functionally significant factor H autoantibodies in two patients with MPGN. Antibody depleting therapy may have a role in such patients and we suggest that screening for factor H autoantibodies should be undertaken in all patients with MPGN.

1. Introduction

Membranoproliferative glomerulonephritis (MPGN) is characterised by thickening of the glomerular basement membrane (GBM) secondary to the deposition of immune complexes, GBM mesangial interposition and hypercellularity. This causes progressive loss of renal function eventually resulting in end stage renal disease (Appel et al., 2005). MPGN in adults and children represents up to 20% and 6.2%, respectively, of all primary glomerulopathies in Europe and North America (Alchi and Jayne, 2010). Children that develop MPGN have a poor prognosis and will likely develop end stage renal disease by early adolescence (Licht et al., 2007). Thus, understanding the mechanisms that drive this disease is important.

Electron microscopy defines three types of MPGN. Type I is characterised by mesangial and subendothelial deposits. It can be idiopathic or associated with hepatitis C, hepatitis B and subacute bacterial endocarditis (West, 1992). Type II, also called dense deposit disease (DDD), is characterized by electron dense deposits within the GBM (Smith et al., 2007). Recent studies have shown that the characteristic EM appearance of DDD is not always associated with the classical light microscopy changes of MPGN (Walker et al., 2007). Type III is characterised by subepithelial deposits with destruction of the GBM (Strife et al., 1984).

Complement activation is seen in all three types (West, 1998). In type I there is activation of both the classical and alternative pathways. In DDD C3 deposition is always seen in the GBM and

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C3 levels alone are consistently low. In type III the glomerular deposits contain C3 but not C4. Complement profiles as with DDD show alternative pathway activation. A circulating factor in MPGN patients which activated C3 *in vitro* was first described in 1969 (Spitzer et al., 1969). This was called C3 nephritic factor (C3NeF) and was subsequently shown to be an IgG autoantibody that stabilises C3bBb (Davis et al., 1977). In addition to C3NeF, a lambda Fv antibody fragment which bound to factor H has been reported in a patient with DDD. The antibody was found to bind to short consensus repeats (SCRs) 1–4, which are part of the regulatory domain of factor H (Jokiranta et al., 1999; Meri et al., 1992).

Factor H autoantibodies are found in ~10% of patients with atypical haemolytic uraemic syndrome (aHUS) (Dragon-Durey et al., 2005, 2010; Jozsi et al., 2007, 2008; Moore et al., 2010). These antibodies block the C-terminal recognition domain of factor H (Jozsi et al., 2007) an area where aHUS associated mutations cluster (Saunders et al., 2006). Most aHUS patients with factor H autoantibodies have complete deficiency of factor H related proteins 1 and 3 (Jozsi et al., 2008; Moore et al., 2010) secondary to a homozygous deletion incorporating *CFHR1* and *CFHR3*. In this study we have examined the hypothesis that functionally significant factor H autoantibodies are associated with MPGN. In two patients with MPGN (one type I and one DDD) we found a high titre of functional factor H autoantibodies which bind predominately to the regulatory domain of factor H.

2. Methods

2.1. Subjects

Serum and DNA samples were available from 16 patients with primary idiopathic MPGN (6 type I, 8 type II DDD and 2 type III) (median age 25, range 3–39; 10 male, 6 female); and 100 healthy blood donors (blood donor controls) (median age 43 years, range 18–68; male $n=44$, female $n=56$). The study was approved by the Newcastle and North Tyneside 1 Research Ethics Committee.

2.2. Factor H autoantibody assay

Flexible 96-well plates were coated with 5 μg/ml of purified factor H (Merck Chemicals Ltd, UK) or molar equivalents of factor H fragments, SCRs 1–5 (generated in house using mammalian cell culture) and (SCRs 7–8, 8–15, 19–20; (Ferreira et al., 2009; Schmidt et al., 2008)) or molar equivalents of factor H related protein 1 (generated in house using mammalian cell culture) in pH 7.6 coating buffer (AbDserotec, UK) and incubated overnight at 4 °C. Plates were washed thrice with PBS/Tween 0.01% followed by blocking for 45 min with 200 μl per well of ultrablock (AbDserotec, UK) at room temperature. A duplicate plate was then set up with block solution as a background binding control. After blocking, a 1/50 dilution of sera in PBS/Tween 0.01% was loaded (50 μl) in triplicate onto both plates and incubated for 1–2 h. Plates were washed thrice and then blocked as above. Goat anti-human IgG horse radish peroxidase (HRP, Stratech Scientific, UK) at 1/4000 was then added and incubated for 1 h at room temperature. Plates were then washed twice with PBS/Tween 0.01% and twice with PBS. Tetramethyl benzidine (TMB) standard kinetic solution (ready made, AbDserotec, UK) was then added to each well for 7 min prior to the reaction being stopped using 10% sulphuric acid. Absorbance at an optical density of 450 nm (OD450) was established using a SpectraMax 190 plate reader (MDS Analytical Technologies Limited, UK). Triplicate data were analysed and mean ultrablock only readings subtracted from mean factor H readings to control for non-specific/false positive readings. For the anti-factor H assay, a standard curve was generated from a known positive (Dragon-Durey et al., 2010) (gifted by Dr M. Dragon-Durey) and the OD450 value for the 1/25 dilution given an extended range and arbitrary 100,000 relative units. Linear-regression curve fit analysis was performed using GraphPad Prism (version 3) and RU values calculated for each sample.

2.3. SDS-PAGE and Western blotting

2.3.1. Detection of autoantibody to factor H

Purified factor H (Comptech, Tx, USA) was diluted in solubilising buffer and loaded onto a 10% SDS-PAGE preparative gel. After transfer to nitrocellulose and blocking, the nitrocellulose was cut into 0.5–1 cm wide strips. These strips were then incubated with individual sera samples (1/100 in 5% dried milk/PBS) for 1–2 h at room temperature. After washing as above, bound auto-antibody was detected using goat anti-human IgG-HRP incubated for 1–2 h at room temperature. Blots were then washed twice with PBS/0.1% Tween 20 and with PBS only.

2.3.2. Detection of factor H related proteins 1 and 3

Sera was applied to anti-human serum albumin beads, prior to being diluted 1/20 in solubilising buffer and run out on 10% SDS-PAGE gels, blotted onto nitrocellulose, and blocked with 5% dried milk/PBS. Blots were incubated with either monoclonal antibody C18/3, known to bind both factor H and factor H related protein 1 (1/1000; Santa Cruz, CA, USA) or a rabbit polyclonal raised against CFHR3 (1/5000; kind gift from Prof P. Zipfel, Jena, Germany) in 5% dried milk/PBS. Blots were washed three times in PBS/0.1% Tween 20 and subsequently incubated with goat anti-mouse-HRP or goat anti Rabbit-HRP, as appropriate (1/3000 in 5% dried milk/PBS). After 1–2 h at room temperature, blots were washed twice with PBS/0.1% Tween 20 and with PBS only.

All blots were developed using an enhanced chemiluminescence (ECL) substrate (Pierce, Cramlington, UK) according to the manufacturer's specifications.

2.4. Purification of IgG and measurement of associated factor H

500 μl of serum from patients and normal subjects was diluted 1 in 4 in PBS before being applied to a 5 ml Protein A/G column (Pierce, UK). After 10 column washes with PBS (containing 0.05% Tween 20), bound IgG was eluted using 0.1 M glycine pH2.5 into 1 M Tris–HCl. Samples were pooled, concentrated and dialysed into PBS and then analysed by standard capture ELISA using OX24 (5 μg/ml) as coat and goat anti-human factor H (1/10,000 Comptech, USA) and donkey anti-goat HRPO (1/10,000) to detect. Samples and control proteins (1 mg/ml factor H) were added in double dilutions and relative concentration established. Concentrated samples were buffer exchanged into alternative pathway buffer (APB or complement fixation buffer (Oxoid, UK) containing 2 mM EGTA and 1.4 mM $MgCl_2$) prior to haemolysis assays.

2.5. Alternative pathway assay

Rabbit Red cells (TCS biological) were washed several times in PBS and subsequently APB. Cells were re-suspended at 0.5% (v/v) and 100 μl plated out on round bottomed 96 well plates containing 100 μl of triplicate serial dilutions of normal human serum in APB either in the presence or absence of 10 μg/ml patient Ig or normal control Ig. Plates were then incubated at 37 °C for 1hr before red cells were pelleted at 500 g for 5 min and supernatant removed. Absorbance at OD410 was used to determine the amount of haemoglobin released in the supernatant from each well. No serum, serum only (no cells) and dH_2O (total lysis) were used as standard controls.

2.6. Complement assays

C3 and C4 levels were measured by rate nephelometry (Beckman Array 360). Factor H and factor I levels were measured by radioimmunodiffusion (Binding Site, Birmingham, UK). The normal ranges were C3 (0.68–1.80 g/l), C4 (0.18–0.60 g/l), factor H (0.35–0.59 g/l) and factor I (38–58 mg/l).

C3NeF was measured using an assay which measured the ability of C3NeF to prolong the half-life of the C3 convertase complex. In brief, C3bBbP was formed on a C3b-coated ELISA plate by incubating patient serum diluted 1/50 in assay buffer (2.5 mM sodium barbitone pH 7.4, 71 mM NaCl, 0.15% Tween, 1 mM $MgCl_2$) with additional 1 μg/ml fB, 0.2 μg/ml fD and 0.5 μg/ml properdin. After 30 min incubation, plates were washed in assay buffer and residual Bb detected using polyclonal goat anti fB (Comptech, Tyler, USA) and HRP-conjugated donkey anti-sheep Ig (Stratech Scientific, diluted 1:5000). Colour was developed using O-phenylenediamine dihydrochloride (Sigma–Aldrich) and measured at 492 nm. Due to the short half-life of Mg^{2+}-stabilised convertase, only C3bBb stabilised by C3NeF gave a positive signal in the assay. Assay blank was obtained by omitting fB and serum/Ig; a sample known to be strongly positive for C3NeF was used as a positive control and results were expressed relative to this: percentage of stabilization = 100× (A492 test sample − A492 blank)/(A492 C3NeF positive control − A492 blank). Samples above the assay cut off (mean of signal from healthy controls + 2SD) were considered positive.

2.7. Mutation screening and measurement of CFHR3/1 copy number

Mutation screening of *CFH*, *CD46*, *CFI*, *CFB*, *C3*, *THBD* and *CFHR5* was undertaken as described previously (Fremeaux-Bacchi et al., 2008; Kavanagh et al., 2005, 2006; Richards et al., 2001, 2003). *CFHR1* and *CFHR3* copy number was measured using multiplex ligation-dependent probe amplification (Schouten et al., 2002) (MLPA).

3. Results

3.1. Factor H autoantibodies

The median (range) antibody titre (RU) in the blood donor controls was 121 (0–3104). Because of the non-normal distribution of antibody titre in the blood donor controls we used the 0.975 fractile to determine autoantibody positivity in the MPGN patients and derived a threshold of 624 RU. We found that 4 MPGN patients had an antibody titre greater than this. In two (P1 and P2) the titres were high whilst in the other two (P3 and P4) they were borderline (Fig. 1a). The specificity of the autoantibodies was confirmed in P1 (MPGN I) and P2 (MPGN II DDD) by Western blotting (Fig. 1b). As previously reported one normal control had detectable factor H autoantibodies (Dhillon et al., 2010; Moore et al., 2010). We were unable to confirm binding in P3 and P4. We were able to obtain additional archived samples from P1 and P2, which showed that factor H autoantibody was present in earlier samples and that the titre had increased in both patients over time (Fig. 1c).

3.2. Clinical details of Patients 1 and 2

Patient 1 is a female who presented at the age of 16 with nephritic syndrome. A renal biopsy showed features consistent with type I MPGN (Fig. 3a–c). Despite steroid treatment renal function progressively deteriorated and 3 years later she commenced dialysis. Nine months later she received a cadaver renal transplant. Whilst initial function was satisfactory there was a progressive decline in transplant function. Six months post transplant a biopsy

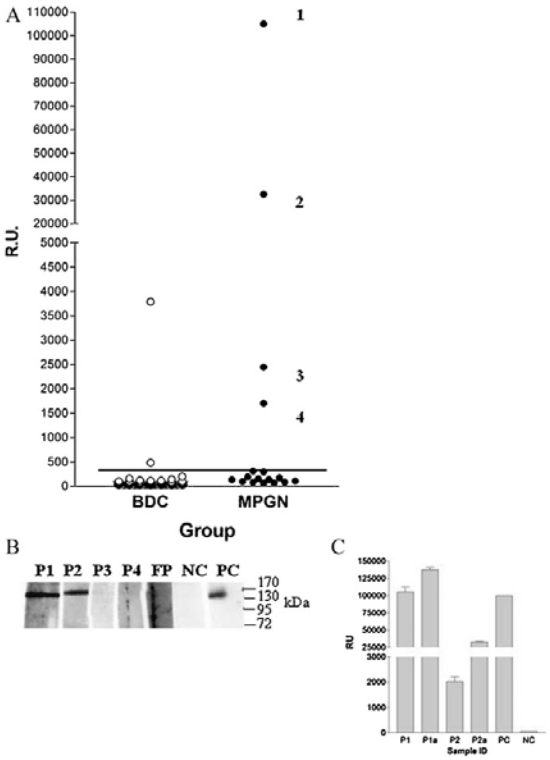

Fig. 1. Detection of factor H autoantibodies. (A) Antibodies against purified factor H in MPGN patients were detected using ELISA as described in the methods section. Serum samples from 100 normal subjects (or blood donor controls, BDC) and 16 MPGN patients were analysed and non-specific background signal subtracted. A standard curve was generated from a known positive sample which was given an arbitrary titer of 100,000 RU from a 1/25 dilution. The horizontal bar represents the cut off for positivity based on the 97.5 percentile of the control cohort (350RU). (B) Purified factor H was run out on 10% SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were then incubated with sera collected from subjects and bound antibody detected. ECL western blotting substrate was used to visualize bound antibody. MPGN patients (P1-4) and sera from known factor H autoantibody positive (positive control, PC), negative (negative control, NC) and high background/false positive (FP) subjects were used as controls. Molecular weight markers are shown and the data is representative of multiple experiments. (C) Illustrates the levels of factor H auto antibody detectable in samples collected from MPGN patients 1 and 2 (P1, P2) on different dates, known positive and negative controls are also shown (PC and NC, respectively).

of the allograft showed recurrent MPGN. One month later she recommenced haemodialysis. Four years later she received a second cadaver transplant. Within one year this too failed due to biopsy proven recurrent disease despite treatment with plasma exchange and rituximab. Complement investigations shortly after her second renal transplant showed C3 0.75 g/l, C4 0.17 g/l, factor H 0.45 g/l and factor I 34 mg/l. C3Nef was negative. A positive titre of factor H autoantibodies was first detected at this time, three years later the titre was at a similar level. Mutation screening of *CFH*, *CD46*, *CFI*, *CFB*, *C3*, *CFHR5* and *THBD* has shown no abnormality. MLPA showed 2 copies of *CFHR1* and *CFHR3* with no evidence of any genomic disorders affecting the genes encoding the factor H related proteins, confirmed at the protein level (Fig. 2a and b).

Patient 2 is a male who presented with advance renal failure at the age of 20. A renal biopsy was undertaken and showed dense deposit disease (Fig. 3d–f). He was treated with dialysis and

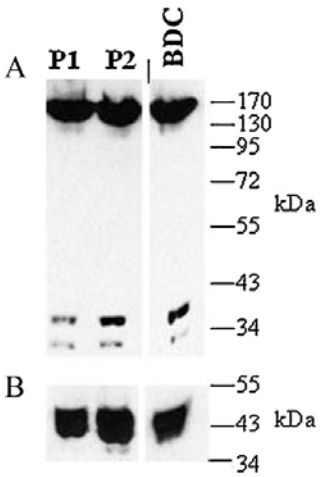

Fig. 2. Western Blot analysis showing normal levels of CFHR1 and CFHR3 in P1 and P2 HSA subtracted patient (P1 and P2) or blood donor control (BDC) sera, were run out on 10% SDS-PAGE and electroblotted to nitrocellulose. Nitrocellulose was then probed with (A) monoclonal antibody C18/3 (detects both CFH and CFHR1) or (B) rabbit anti CFHR3 as outlined in methods. Molecular weight markers are shown on all and the data are representative of multiple experiments.

2 years later received a live-related transplant from his mother. Initial graft function was good but a renal biopsy undertaken at 3 months because of a rising creatinine demonstrated recurrent dense deposit disease and despite treatment with plasma exchange and rituximab the graft failed 18 months after transplantation. Complement investigations at this time showed C3 0.29 g/l, C4 0.27 g/l, factor H 0.72 g/l and factor I 75 mg/l. C3NeF was strongly positive. A positive titre of factor H autoantibodies was detected on samples taken both at this time and one year later. Mutation screening of *CFH*, *CD46*, *CFI*, *CFB*, *C3*, *CFHR5* and *THBD* has shown no abnormality. MLPA showed 2 copies of *CFHR1* and *CFHR3*.

3.3. Binding of autoantibodies to factor H

The site of binding of the autoantibodies was determined using factor H fragments (SCRs 1–5, 7–8, 8–15, 19–20) and recombinant factor H related protein 1. Antibodies from both P1 and P2 showed strong binding to factor H SCR1-5 fragment (Fig. 4a). However, antibodies from P2 also showed binding to SCR7-8 and SCR8-15 fragments. There was no binding to factor H SCR 19–20 or factor H related protein 1. P3/4 did not strongly bind to any particular fragment in this analysis.

3.4. A small percentage of patient factor H can be isolated as an immune complex

We applied patient or normal control sera to a protein A/G column and collected total Ig. Greater than 95% of total Ig was purified from the serum samples after one run over the column (data not shown). Analysis of pre and post column fractions of serum showed that a small proportion (~5%) of available factor H was tightly bound or co-purified with patient Ig which was higher than that seen in the normal control (≤0.1%) but not as much as found in an aHUS patient with high titre factor H autoantibodies (~25%; Fig. 4b). Factor H eluted from the column for a protracted period during washing. This was particularly evident in patients with autoantibodies.

3.5. Purified patient Ig interferes with factor H function in an alternative pathway haemolytic assay

Using an alternative pathway haemolytic assay we determined whether purified Ig from the MPGN patients interfered with the normal function of factor H. We found that Ig isolated from both P1 and P2 increased lytic activity (Fig. 4c).

4. Discussion

In this study we have identified 2 MPGN patients with high titre factor H autoantibodies. P1 in this study has one of the highest autoantibody titres that we have observed in other diseases including aHUS and age-related macular degeneration (Dhillon et al., 2010; Moore et al., 2010). There have recently been other reports of the presence of factor H autoantibodies in patients with MPGN (Brackman et al., 2011; Lorcy et al., 2011; Sethi et al., 2011) but we have in addition examined both the binding site and functionality of the autoantibodies.

In aHUS factor H autoantibodies bind predominantly to the C terminal recognition domain (Jozsi et al., 2007; Moore et al., 2010) and impair the regulatory function of factor H at the cell surface. This region of factor H is where aHUS-associated mutations cluster (Saunders et al., 2006). In the two MPGN patients described here the antibodies bind the N terminal regulatory domain. This is compatible with the paradigm that fluid-phase dysregulation of the alternative pathway predisposes to glomerulonephritis whereas dysregulation at the cell surface predisposes to a thromobotic microangiopathy. In aHUS the presence of factor H autoantibodies is associated with complete deficiency of factor H related proteins 1 and 3 (Dragon-Durey et al., 2010; Jozsi et al., 2008; Moore et al., 2010). In this study both of the MPGN patients with factor H autoantibodies had two copies of both *CFHR1* and *CFHR3*.

MPGN is known to be associated with homozygous factor H deficiency. Pickering and Cook reviewed the clinical and laboratory investigations of 33 factor H deficient individuals from 15 families (Pickering and Cook, 2008). Nine of these were reported to have MPGN. Of these 4 (including one sib pair) were found to have a homozygous *CFH* mutation. MPGN is also associated with homozygous factor H mutations affecting the regulatory domain. Licht et al. reported the finding of type II MPGN in two sibs of a consanguineous family in association with a homozygous *CFH* mutation in the exon encoding SCR 4 (Licht et al., 2006). This resulted in the deletion of a single lysine residue but the mutant protein was secreted and showed severely reduced cofactor and decay-accelerating activity, as well as reduced binding to C3b. All the individuals carrying this change were also C3NeF positive. It has been shown that type I MPGN can sometimes be associated with isolated C3 deposition. In this subgroup heterozygous mutations in *CFH*, *CFI* and *CD46* have been reported (Servais et al., 2007). Familial DDD is rare but in a unique pedigree it has been shown that a *C3* mutation (c.2767_2774delACGGTG) results in a mutant ($C_{3923\Delta DG}$) which in its hydrolysed C3b form is resistant to proteolysis by factor I in the presence of factor H but not CD46 (Martinez-Barricarte et al., 2010). In patients with aHUS-associated factor H autoantibodies we have found that ~40% of patients also carry a mutation in *CFH*, *CFI*, *CD46*, and *C3*. In the two MPGN patients described here we screened *CFH*, *CFI*, *CD46*, C3, *CFB*, *CFHR5* and *THBD* and did not find any abnormalities. Neither did we detect on screening with MLPA any evidence of a genomic disorder affecting the genes encoding the factor H related proteins as has been described in CFHR5 nephropathy (Gale et al., 2010). However, in P2 with DDD, we in addition to finding factor H autoantibodies also detected C3NeF. The association of C3NeF with MPGN and in particular DDD is well established (Licht and

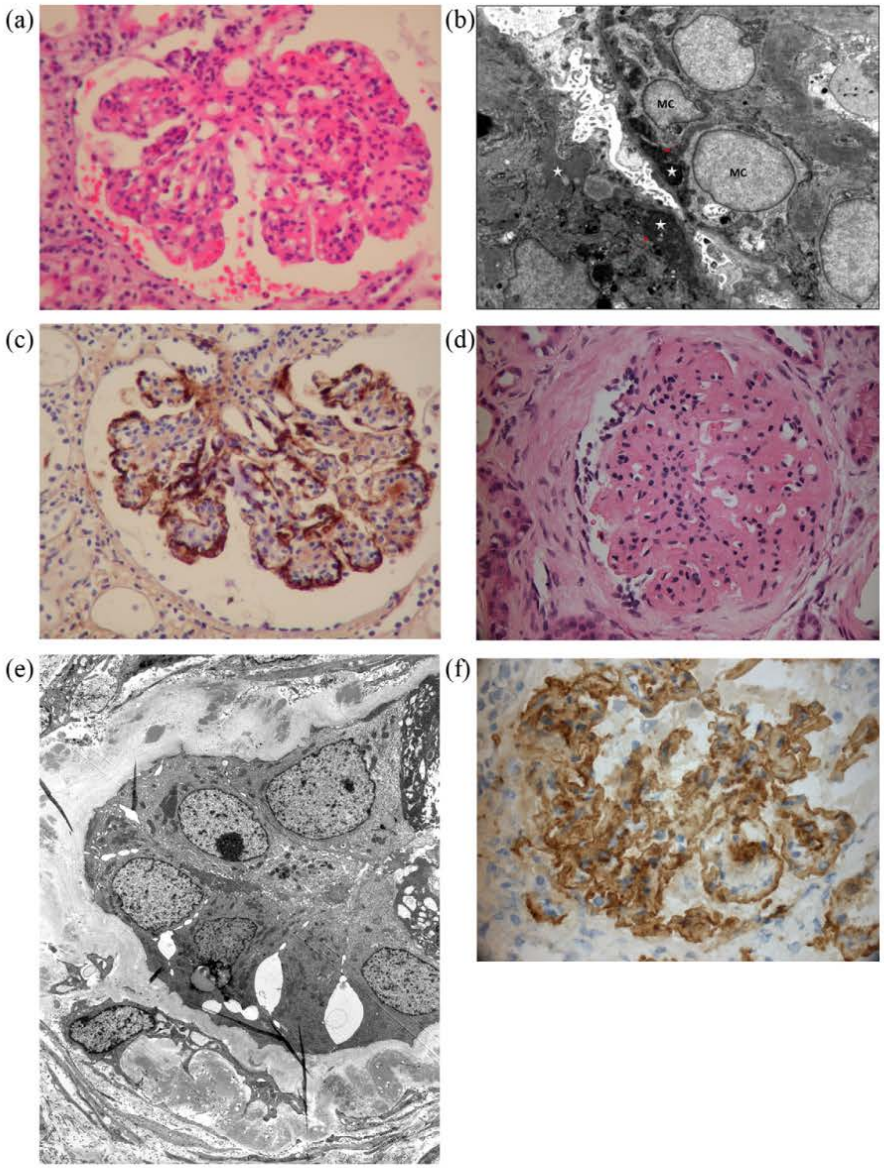

Fig. 3. Renal biopsies at presentation. (a) Light microscopy from patient 1 showing lobular architecture with mesangial expansion and mesangial cell proliferation. Stained with H&E, magnification ×400. (b) Electron micrograph from patient 1 showing expanded hypercellular mesangium with electron dense deposits (*) and mesangial cell (MC) interposition, magnification ×2900. (c) Light microscopy from patient 1 stained for C3 with immunoperoxidase showing global C3 deposition within the capillary walls, magnification ×400. (d) Light microscopy from patient 2 showing global capillary wall thickening by eosinophilic hyaline material. The mesangium is expanded by eosinophilic material and there is segmental mesangial hypercellularity. Stained with H&E, magnification ×400. (e) Electron micrograph from patient 2 showing broad discontinuous electron dense deposits within the lamina densa, producing fusiform thickening of the glomerular basement membrane. (f) Light microscopy from patient 2 stained for C3 with immunoperoxidase showing interrupted linear staining of the glomerular capillary walls and granular staining of the mesangium for C3, magnification ×400.

Fremeaux-Bacchi, 2009; Smith et al., 2007). The origin of C3NeF is unclear (Spitzer et al., 1992) but as with factor H autoantibodies in aHUS it has been shown that some MPGN patients with C3NeF also carry mutations in *CFH* and *CFI* (Leroy et al., 2011; Licht et al., 2006). Factor B autoantibodies on their own and in combination with C3b autoantibodies have also been described in association with DDD (Chen et al., 2011; Strobel et al., 2010).

We have undertaken functional analysis of the factor H autoantibodies purified from these two MPGN patients using haemolytic assays. The presence of C3NeF in P2 confounds the interpretation

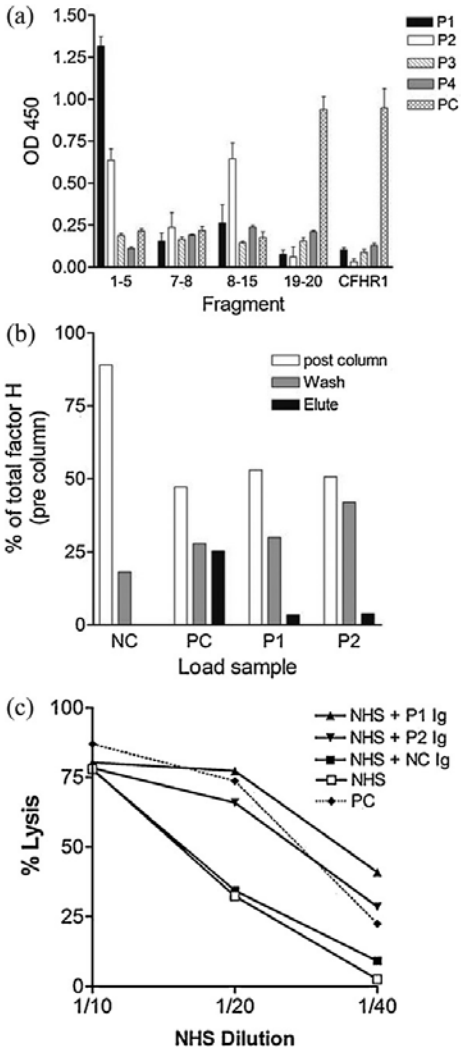

Fig. 4. Binding site and functional significance of the factor H autoantibodies. (a) Determination of binding site with factor H. MPGN patient sera was applied to ELISA plates coated with purified recombinant factor H (SCRs 1–5, 7–8, 8–15, 19–20) fragments or factor H related protein 1 (CFHR1) or blocked plate only as described in the methods section. Non-specific background signal was subtracted and the signal for each protein/fragment is shown. P1, P2, P3 and P4 are the MPGN patients. PC is a positive control sample from an aHUS patient known to have a high titre factor H autoantibody. Results shown are mean plus standard deviation from at least 3 independent experiments standardized on the negative control. (b) A proportion of patient factor H is isolated as an immune complex. Serum samples were applied to and eluted from a protein A/G column according to manufacturer's instructions (Thermo). Pre, Post, wash and eluted fractions were then applied to a factor H specific ELISA. As individual levels of factor H varied, the levels of factor H in each fraction are shown as a percentage of the pre column load value (100%) for each patient or control. NC is a normal healthy blood donor. P1, P2 and PC are as previously. Data is representative of two such experiments. (c) Functional properties of factor H autoantibodies. Alternative pathway mediated rabbit red cell lysis by normal human sera (NHS) in the presence or absence of isolated IgG (Ig) was measured. As control, the level of lysis (released heamoglobin measured at OD 410) seen in the presence (NC) or absence (NHS) of purified Ig isolated from a healthy blood donor is also shown. Where added, the final concentration of purified patient or control IgG was equivalent to 100 μg/ml (or 10 μg per well) P1, P2 and PC are as noted above. The results shown are representative of three comparable experiments.

of the results (Rother, 1982) but in both patients there is clear evidence of increased lysis.

In summary we have detected factor H autoantibodies in two patients with MPGN. Whilst functionally significant it is difficult to determine whether the antibodies are disease-causing or disease-associated. Further studies in larger cohorts of MPGN patients will help to elucidate this.

Acknowledgment

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Publication 4

4. Holmes *et al*., (2012) Determining the population frequency of the CFHR3/CFHR1 deletion in the regulation of complement activation (RCA) gene cluster at 1q32 (*publication under review at time of printing thesis*).

Contributions

Tables 2-4: I conducted the analysis of MLPA results to determine *CFHR3/1* copy number within the UK control cohort. This data set is not included in this thesis.

Determining the population frequency of the *CFHR3/CFHR1* deletion in the regulation of complement activation (RCA) gene cluster at 1q32

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Abstract

In this study we have used multiplex ligation-dependent probe amplification (MLPA) to measure the copy number of *CFHR3* and *CFHR1* in DNA samples from 238 individuals from the UK and 339 individuals from the HGDP-CEPH Human Genome Diversity Cell Line Panel. We have then calculated the allele frequency and frequency of homozygosity for the copy number polymorphism represented by the *CFHR3/CFHR1* deletion. There was a highly significant difference between worldwide populations in both the allele frequency (X^2=127.7, DF=11, P-value=4.97x10^{-22}) and frequency of homozygosity (X^2=142.3, DF=22, P-value=1.33x10^{-19}). The highest allele frequency (54.7%) was seen in DNA samples from Nigeria and the lowest (0%) in samples from South America and Japan. The association of the deletion with AMD, SLE and IgA nephropathy is in keeping with differences in the prevalence of these diseases in worldwide populations. This emphasises the importance of identifying copy number polymorphism in disease.

Introduction

Complement genes within the RCA (Regulators of Complement Activation) cluster at chromosome 1q32 are arranged in tandem within two groups [1]. In a centromeric 360 kb segment lie the genes for factor H (*CFH*) (OMIM 134370) and five factor H-related proteins – *CFHR1* (OMIM 134371), *CFHR2* (OMIM 600889), *CFHR3* (OMIM 605336), *CFHR4* (OMIM 605337) and *CFHR5* (OMIM 608593). Sequence analysis of this region shows evidence of segmental duplications (SDs) resulting in a high degree of sequence identity between *CFH* and the genes for the five factor H related proteins [2-4]. SDs such as those seen in the RCA cluster are frequently associated with genomic rearrangements [5]. These usually occur as a result of non-allelic homologous recombination (NAHR) between SDs but can also be a result of gene conversion and microhomology mediated end joining (MMEJ)[6]. Genomic disorders at this locus have affected *CFH* and the *CFHR*s in a number of ways. Deletions as a result of NAHR lead to the loss of *CFHR1*, *CFHR3* and *CFHR4*. Deletions within genes, occurring through both NAHR and MMEJ, result in the formation of hybrid genes (*CFH/CFHR1*, *CFHR1/CFH*, *CFH/CFHR3*, *CFHR3/CFHR1*) associated with diseases such as atypical haemolytic uraemic syndrome (aHUS) and membranoproliferative glomerulonephritis (MPGN) [7-9]. Gene conversion between *CFH* and *CFHR1* has resulted in aHUS associated factor H mutants [10] and allelic variants of *CFHR1* [11]. The *CFHR3/1* deletion is now accepted to be a copy number polymorphism (CNP) because it is present in populations at a frequency of >1%. It has been extensively characterised in health and disease. In 1992 proteomic studies were the first to suggest that complete deficiency of factor H related proteins 1 and 3 was a polymorphic variant present in ~ 4% of a European population [12]. That NAHR was the molecular mechanism responsible for this was recognised in studies showing that the deletion was associated with a decreased risk of age-related macular degeneration [13]. Subsequently it has been shown that the deletion is associated with the presence of factor H autoantibodies in aHUS [14,15], with an increased risk of SLE [16] and a decreased risk of IgA nephropathy [17,18]. That there might be differences in the population frequency of the *CFHR3/1* deletion was suggested from a study published in 2006 which showed that the prevalence of homozygous deletion in African populations was ~16% [19]. Populations difference in the deletion have been confirmed in subsequent studies [16,20,21] In this study we have

measured copy number of *CFHR3* and *CFHR1* with multiplex ligation-dependent probe amplification (MLPA) [22] in a range of populations derived from the HGDP-CEPH Human Genome Diversity Panel (http://www.cephb.fr/en/hgdp/diversity.php) [23].

Methods

CFHR1 and *CFHR3* copy number was measured in DNA samples from 238 individuals from the *UK* and 439 individuals from the HGDP-CEPH panel. The UK samples comprised 70 DNA samples from the Health Protection Agency Culture Collections (http://www.hpacultures.org.uk/products/dna/hrcdna DNA), 10 samples obtained from local blood donors and 158 DNA samples from control individuals within the Wellcome Trust Case Control Consortium [24,25]. The samples from the Health Protection Agency were originally obtained from a control population of randomly selected, non-related UK Caucasian blood donors. The full collection of samples with the HGDP-CEPH panel consists of 1050 individuals from 51 world populations (http://www.cephb.fr). We selected for analysis 439 samples from 17 different populations (Table 1). We did not include populations for which data is either already available (for example European populations such as France) or where samples numbers were too small to be representative. There were still some populations with a small number of samples but many come from the sub-Saharan region of Africa and were considered together in subsequent analysis. In total 133 samples from African populations were analysed, including 83 from sub-Saharan countries. *CFHR1* and *CFHR3* copy number was measured as described previously [26] using multiplex ligation-dependent probe amplification [22](MLPA) using a kit from MRC Holland (www.mlpa.com) (SALSA MLPA kit P236-A1 ARMD) and in house probes.

Statistics

Chi-square analysis was undertaken to determine whether there was a significant difference between populations in either the allele frequency of the *CFHR3/CFHR1* deletion or the frequency of a homozygous deletion of *CFHR3/CFHR1*. Fisher's exact test were undertaken to determine whether in individual populations either the allele frequency of the *CFHR3/CFHR1* deletion or the frequency of a homozygous deletion of *CFHR3/CFHR1* was significantly different to the values for these variables in the UK population.

Results

The allele frequency of the *CFHR3/CFHR1* deletion in the various populations is shown in table 2. The *CFHR3/CFHR1* deletion was not present in either the South American or Japanese populations. The highest allele frequency for the deletion was 54.7% in Nigeria. The deletion was present in all other populations studied, with allele frequencies of 1.5%, 2.1% and 6.0% in Mexico, Siberia and China respectively, 15% in Pakistan, 18.3% in the UK, 22.4% in Italy and North Africa, 25.6% in Russia and 33.7% in Sub-Saharan Africa. Differences between populations were highly significant (X^2=127.7, DF=11, P-value=4.97x10^{-22}). The level of statistical significance derived using Fishers exact test for the allele frequency of the *CFHR3/CFHR1* deletion in individual populations compared to the UK population is shown in table 2. The allele frequency of the *CFHR3/CFHR1* deletion was significantly lower in South America, Japan, Mexico, Siberia and China; was not significantly different in Pakistan, Italy, North Africa and Russia but was significantly higher in sub-Saharan Africa and Nigeria.

The *CFHR3/CFHR1* deletion was not found in homozygosity in Mexico, South America, China, Japan, Pakistan or Siberia. The frequency of homozygous deletion was 3.4% in the UK, between 5-10% in Italy, Russia, North Africa and Sub-Saharan Africa, and 33.3% in Nigeria. Differences between populations were highly significant (X^2=142.3, DF=22, P-value=1.33x10^{-19}). The level of statistical significance derived using Fishers exact test for the freqeuency of the *CFHR3/CFHR1* deletion in homozygosity in individual populations compared to the UK population is shown in table 3. The frequency was significantly lower in South America, Japan, Mexico, Siberia and China; was not significantly different in Pakistan, Italy, North Africa and Russia but was significantly higher in sub-Saharan Africa and Nigeria.

Discussion

In this study we have used multiplex ligation-dependent probe amplification (MLPA) to determine the copy number of *CFHR3* and *CFHR1* in a variety of different populations derived from the HGDP-CEPH collection. From this we have calculated the allele frequency of the *CFHR3/CFHR1* deletion. We have used MLPA because this provides a specific determination of copy number. We have

measured copy number of both *CFHR3* and *CFHR1* to determine the allele frequency of the *CFHR3/CFHR1* deletion because measurement of *CFHR1* copy number alone to determine this is compounded by the *CFHR1/CFHR4* deletion [11,26]. Using MLPA we have been able to determine both the allele frequency of the deletion and the frequency of a homozygous deletion. For statistical purposes we have set the UK population as our reference. The value of 3.4% for the frequency of a homozygous deletion in the UK population in this study is similar to values that we have obtained in previous studies [14,26] (Table 4). The allele frequency of the deletion we have calculated in previous studies to be 6.3% in Zipfel et al[14] and 17.3% in Moore et al [26]. The latter value is similar to the frequency of 18.3% that we have obtained in this study. We believe that sample size may have played a role in the discrepancy seen between the three studies. In the study of Zipfel et al [14] we studied 119 individuals, the chi-squared test p value for the "genotype" distribution in this study was 0.06 compared to 0.99 for the study of Moore et al [26] where we studied 505 individuals. Thus the lower the number of individuals in the reference control population the greater the risk of obtaining "genotype" frequencies that are not compatible with Hardy-Weinberg equilibrium. In this study we analysed 238 individuals in the UK control group and the chi-squared test p value for the "genotype" distribution was 0.99.

The values for the allele frequency of the deletion and the frequency of a homozygous deletion that we obtained for world-wide populations using the HGDP-CEPH collection show marked population differences with the highest frequencies being seen in African populations particularly the Nigerian one. These results are consistent with those obtained initally by Hageman et al [19] in African Americans and HGDP-CEPH African samples. Hageman, however, used a gene specific PCR method which only allowed measurement of the frequency of a homozygous deletion. Subsequently there have been several other studies documenting the frequency of the *CFHR3/CFHR1* deletion in a range of populations. The results from these are shown in table 4. The values that we obtained in this study for both allele frequency and the frequency of homozygous deletion are consistent with previous studies particularly for the UK, Japanese, Chinese and Nigerian populations. We chose in this study because of low numbers to combine several populations from sub-saharan Africa. The study of Sivakumaran et al [21] suggests that for this region there are significant differences in the allele

frequency of the deletion between tribes. For instance, they found an allele frequency of 23.8% in the Maasai tribe of Kenya compared to 42.3% in the Luhya.

How in evolution has this deletion arisen and how can the population differences be explained? The alternative pathway of complement is thought to be the oldest component of the innate immune system [27]. The earliest components of the alternative complement pathway to have been recognised are activators such as C3 which has been identified in a coral [28] suggesting their presence in the Cnidria. Regulatory components have been first recognised in the Agnatha with for instance identification of a C3 cleaving short consensus repeat protein in lamprey [29]. A protein (called SBP1) with a high degree of homology to human factor H was first described in the teleost, sand bass [30,31]. Factor H has also been identified in the Zebrafish [32]. In the zebrafish, the mouse and humans there are genes encoding SCR proteins with a high degree of homology to factor H in close proximity to the gene encoding factor H. In man there are the five factor H related proteins (*CFHR1-5*), in the mouse there are three factor H related proteins and in the zebrafish there are 4 factor H like proteins. Sequence analysis of this region in man suggests that these genes have arisen through a series of segmental duplication events [2]. Analysis of primate genomes undertaken by Sivakumaran et al suggests that chimps have more extensive duplication in this region than humans. The analysis also suggests that the duplications arose in a common ancestor of the chimp and humans after divergence from the orang-utan [21]. The duplicated segments predispose to both non-allelic homologous recombination (NAHR) and gene conversion [33]. The available evidence would suggest that the *CFHR3/CFHR1* deletion has arisen through NAHR after the initial formation of the SDs. We believe that in certain populations that the deletion has resulted in an evolutionary benefit. The role of complement in diseases such as malaria [34] would suggest that is driven by infection.

Since the *CFHR3/CFHR1* deletion was first described a number of studies have documented strong linkage disequilibrium of the deletion with common *CFH* haplotypes [35,36]. In some populations the deletion is present on haplotypes H1-5 and absent on H6-7 [35]. In other populations the H2 haplotype perfectly tags the deletion [17]. Likewise in some populations individual SNPs have been shown to be in complete LD with the deletion. Zhao et al found that the deletion was in complete LD in European Americans with rs6677604 but not African Americans ($r^2 = 0.60$). Whether the deletion

confers an independent risk for AMD, SLE and IgA nephropathy or is simply associated with protective/at-risk haplotypes is an area of controversy [21,35,37]. However, factor H related protein 1 blocks the C5 convertase but binds, in competition with factor H, to host surfaces through its c terminal regulatory domain [38]. We are, therefore, of the opinion that deletion of *CFHR1* has a dual effect with reduced inhibition of terminal complement pathway activity but increased regulation by factor H of the alternative pathway. This may also explain why in some diseases (AMD and IgA nephropathy) the deletion is protective whilst in other others (SLE) it is associated with increased risk. Figures for the prevalence of these diseases in African and European Americans are consistent with the allele frequency of the deletion in these two groups (~38% in African Americans and ~22% in European Americans). In African Americans with a higher frequency of the deletion the prevalence of AMD and IgA nephropathy is lower than in European Americans[39,40] whereas the prevalence of SLE is higher [41]. Thus studying the population frequency of disease associated CNPs such as the *CFHR3/CFHR1* deletion provides novel insights into the pathogenesis of such diseases.

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Table 1. HGDP-CEPH samples used for measurement of *CFHR3* and *CFHR1* copy number.

Geographical location	Number of samples analysed
Algeria	29
Brazil	22
Central African Republic	23
China	50
Columbia	7
Democratic Republic of Congo	13
Italy	49
Japan	29
Kenya	11
Mexico	34
Namibia	6
Nigeria	21
Pakistan	50
Russia	41
Senegal	22
Siberia	24
South Africa	8
TOTAL	439

Table 2. Allele frequencies of the *CFHR3/CFHR1* deletion in UK and HGDP-CEPH populations. The P value derived using Fisher's exact test compares the allele frequency of the *CFHR3/CFHR1* deletion in HGDP-CEPH populations to that of the UK population.

Population	Allele frequency of the *CFHR3/CFHR1* deletion (%)	P value compared to the UK population
UK (n=238)	18.3	
South America[1] (n=29)	0.0	3.53×10^{-5}
Japan (n=29)	0.0	3.53×10^{-5}
Mexico (n=34)	1.5	6.75×10^{-5}
Siberia (n=24)	2.1	0.0019
China (n=50)	6.0	0.0153
Pakistan (n=50)	15.0	0.479
Italy (n=49)	22.4	0.326
North Africa[2] (n=29)	22.4	0.476
Russia (n=41)	25.6	0.131
Sub-Saharan Africa[3] (n=83)	33.7	8.37×10^{-5}
Nigeria (n=21)	54.7	5.85×10^{-7}

[1]Brazil, Columbia

[2]Algeria

[3]Central African Republic, Democratic Republic of Congo, Kenya, Namibia, Senegal, South Africa

Table 3. Homozygous *CFHR3/CFHR1* deletion frequencies in UK and HGDP-CEPH worldwide populations. The P value derived using Fisher's exact test compares the frequency of homozygous *CFHR3/CFHR1* deletion in HGDP-CEPH populations with that of the UK population.

Population	Homozygous *CFHR3/CFHR1* deletion (%)	P value compared to the UK population
UK (n=238)	3.4	
South America (n=29)	0.0	1.60×10^{-4}
Japan (n=29)	0.0	1.60×10^{-4}
Mexico (n=34)	0.0	4.63×10^{-4}
Siberia (n=24)	0.0	0.0092
China (n=50)	0.0	0.0094
Pakistan (n=50)	0.0	0.578
Italy (n=49)	6.1	0.470
North Africa (n=29)	6.9	0.425
Russia (n=41)	7.3	0.219
Sub-Saharan Africa (n=83)	8.4	1.14×10^{-4}
Nigeria (n=21)	33.3	2.06×10^{-6}

Table 4. Reported population frequencies of the *CFHR3/CFHR1* deletion

Population	Number of individuals	Allele frequency of *CFHR3/1 del*	*CFHR3/1 del/del*	Method	Reference
UK	119	6.3%	1.6%	MLPA	[14]
UK	505	17.3%	3.0%	MLPA	[26]
France	70	8.6%	2.9%	MLPA	[42]
Spain	129	24%	7.0%	MLPA and WB	[11]
European American	275	19.8%	4.4%	MLPA	[16]
Asian	282	5.7%	0.7%	MLPA	[16]
Hispanic	196	17.8%	2.6%	MLPA	[16]
African American	106	42%	16%	MLPA	[16]
Austria	252		4.4%	WB	[12]
Germany	100		2.0%	WB	[14]
Tunisia	59		20%	WB	[20]
African American	347		15.9%	Gene specific PCR	[19]
Hispanic	266		6.8%	Gene specific PCR	[19]
European American	279		4.7%	Gene specific PCR	[19]
Chinese	94		2.2%	Gene specific PCR	[19]
HGDP African	127		17.3%	Gene specific PCR	[19]
HGDP North African	29		17.2%	Gene specific PCR	[19]
HGDP Middle Eastern	211		14.7%	Gene specific PCR	[19]
HapMap CEU	60	24.2%	8.3%		[21]
HapMap CHB	45	8.9%	0%		[21]
HapMap JPT	45	6.7%	0%		[21]
HapMap YRI	60	55%	28%		[21]
Coriell Diversity Panel African American	100	37%	17%		[21]
Coriell Diversity Panel Caucasian	100	21%	4%		[21]
Coriell Diversity Panel Chinese	100	4.5%	0%		[21]
Coriell Diversity Panel Mexican	100	13%	0%		[21]
HapMap III CEU	59	21.2%	1.7%		[21]
HapMap III TSI	90	24.4%	5.6%		[21]
HapMap III GIH	90	38.3%	18.9%		[21]
HapMap III MEX	55	11.8%	1.8%		[21]
HapMap III CHB	45	6.7%	0%		[21]
HapMap III CHD	50	3.5%	0%		[21]
HapMap III JPT	46	3.3%	0%		[21]
HapMap III ASW	53	34.0%	9.4%		[21]
HapMap III LWK	52	42.3%	19.2%		[21]
HapMap III MKK	149	23.8%	3.4%		[21]
HapMap III YRI	60	53%	27%		[21]

MLPA, multiplex ligation-dependent probe amplification
WB, western blotting
PCR, polymerase chain reaction.
CEU, Utah residents with Northern and Western European ancestry from the CEPH collection
CHB, Han Chinese in Beijing, China
CHD, Chinese in Metropolitan Denver, Colorado
GIH, Gujarati Indians in Houston, Texas
JPT, Japanese in Tokyo, Japan
LWK, Luhya in Webuye, Kenya
MEX, Mexican ancestry in Los Angeles, California
MKK, Maasai in Kinyawa, Kenya
TSI, Toscans in Italy
YRI, Yoruba in Ibadan, Nigeria
ASW, African ancestry in Southwest USA